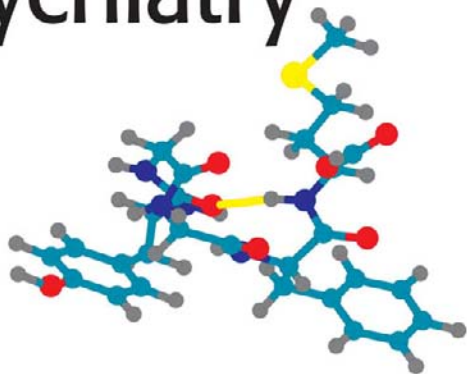


SOLOMON H. SNYDER, M.D.

Science and Psychiatry



**Groundbreaking Discoveries in
Molecular Neuroscience**

Science and Psychiatry

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Molecular Neuroscience

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Science and Psychiatry

Groundbreaking Discoveries in Molecular Neuroscience

By Solomon H. Snyder, M.D.

Foreword by Eric R. Kandel

Commentaries by Eric J. Nestler, Charles B. Nemeroff, George Aghajanian,
Carol A. Tamminga, Herbert Y. Meltzer, Robert M. Post, Anne B. Young,
Joseph T. Coyle, Samuel Barondes, and Nancy C. Andreasen



Washington, DC
London, England

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*To my grandchildren Abigail, Emily, and Leo,
the most groundbreaking discoveries of all.*

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Contents

| | |
|------------------------|------|
| About the Author | xiii |
|------------------------|------|

Foreword

| | |
|---|----|
| Sol Snyder: Audacious Scientist, Leader of the Scientific Community, and Remarkable Human Being! | xv |
| <i>Eric R. Kandel</i> | |

| | |
|--------------------------|-------|
| Introduction | xxiii |
| <i>Solomon H. Snyder</i> | |

Part I

DISCOVERY AND CHARACTERIZATION OF THE OPIATE RECEPTOR

Commentary

| | |
|--|---|
| Radioligand Binding Methodologies: New Inventions, New Directions | 3 |
| <i>Eric J. Nestler</i> | |

1 Opiate Receptor

| | |
|---|---|
| Demonstration in Nervous Tissue | 9 |
| <i>Candace B. Pert, Solomon H. Snyder</i> | |

2 Opiate Agonists and Antagonists Discriminated by Receptor Binding in Brain

| | |
|---|----|
| | 19 |
| <i>Candace B. Pert, Gavril Pasternak, Solomon H. Snyder</i> | |

| | | |
|----------|---|----|
| 3 | Historical Review | |
| | Opioid Receptors | 27 |
| | <i>Solomon H. Snyder, Gavril W. Pasternak</i> | |

Part II

CHARACTERIZATION OF THE ENKEPHALINS

| | | |
|----------|---|----|
| | Commentary | |
| | The Discovery of Endogenous Opiates and Their Receptors: | |
| | A Snyderian Saga of Skill and Judgment. | 49 |
| | <i>Charles B. Nemeroff</i> | |
| 4 | Opiate Receptor in Normal and Drug Altered | |
| | Brain Function | 53 |
| | <i>Solomon H. Snyder</i> | |
| 5 | Morphine-Like Peptides in Mammalian Brain | |
| | Isolation, Structure Elucidation, and Interactions With the | |
| | Opiate Receptor | 69 |
| | <i>Rabi Simantov, Solomon H. Snyder</i> | |
| 6 | Opioid Peptide Enkephalin | |
| | Immunohistochemical Mapping in Rat Central Nervous System. | 81 |
| | <i>Rabi Simantov, Michael J. Kuhar, George R. Uhl,</i> | |
| | <i>Solomon H. Snyder</i> | |

Part III

DOPAMINE RECEPTORS AND INFLUENCES OF NEUROLEPTICS

| | | |
|----------|---|-----|
| | Commentary | |
| | Dopamine Receptor Binding and Its Therapeutics. | 97 |
| | <i>George Aghajanian</i> | |
| 7 | Dopamine Receptor Binding Predicts Clinical and | |
| | Pharmacological Potencies of Antischizophrenic Drugs | 103 |
| | <i>Ian Creese, David R. Burt, Solomon H. Snyder</i> | |

- 8 Antischizophrenic Drugs**
 Chronic Treatment Elevates Dopamine Receptor Binding in Brain 113
David R. Burt, Ian Creese, Solomon H. Snyder
- 9 Dopamine Receptors, Neuroleptics, and Schizophrenia 121**
Solomon H. Snyder

Part IV

DRUG EFFECTS EXPLAINED AS ACTIONS ON NEUROTRANSMITTER RECEPTORS

- Commentary**
 Perspectives on Simplicity and Discovery 133
Carol A. Tamminga
- 10 Antischizophrenic Drugs and Brain Cholinergic Receptors**
 Affinity for Muscarinic Sites Predicts Extrapyramidal Effects 137
Solomon Snyder, David Greenberg, Henry I. Yamamura
- 11 Tricyclic Antidepressants**
 Therapeutic Properties and Affinity for α -Noradrenergic Receptor
 Binding Sites in the Brain 147
*David C. U'Prichard, David A. Greenberg,
 Peter P. Sheehan, Solomon H. Snyder*

Part V

DRUG ACTIONS AND SEROTONIN RECEPTOR SUBTYPES

- Commentary**
 Clinical Data Stimulation of Basic Research: The Far-Reaching Clinical
 Significance of Serotonin Receptor Subtype Identification 157
Herbert Y. Meltzer
- 12 Long-Term Antidepressant Treatment Decreases**
Spiroperidol-Labeled Serotonin Receptor Binding 163
Stephen J. Peroutka, Solomon H. Snyder

- 13** Two Distinct Central Serotonin Receptors With Different Physiological Functions171
*Stephen J. Peroutka, Richard M. Lebovitz,
Solomon H. Snyder*

Part VI

INOSITOL PHOSPHATES AND ACTIONS OF LITHIUM

Commentary

- Lithium, Second Messengers, and Downstream Effects181
Robert M. Post

- 14** Lithium Blocks a Phosphoinositide-Mediated Cholinergic Response in Hippocampal Slices189
*Paul F. Worley, William A. Heller, Solomon H. Snyder,
Jay M. Baraban*

- 15** Solubilization, Purification, and Characterization of an Inositol Trisphosphate Receptor195
*Surachai Supattapone, Paul F. Worley, Jay M. Baraban,
Solomon H. Snyder*

- 16** Second Messenger Systems and Psychoactive Drug Action
Focus on the Phosphoinositide System and Lithium209
Jay M. Baraban, Paul F. Worley, Solomon H. Snyder

- 17** Purified Inositol 1,4,5-Trisphosphate Receptor Mediates Calcium Flux in Reconstituted Lipid Vesicles231
*Christopher D. Ferris, Richard L. Haganir,
Surachai Supattapone, Solomon H. Snyder*

Part VII

NITRIC OXIDE AS A NEUROTRANSMITTER

Commentary

- Just Say "Yes": Snyder's Approach to the Difficult Problem of NO243
Anne Young

| | | |
|-----------|--|-----|
| 18 | Nitric Oxide Mediates Glutamate-Linked Enhancement of cGMP Levels in the Cerebellum | 249 |
| | <i>David S. Bredt, Solomon H. Snyder</i> | |
| 19 | Cloned and Expressed Nitric Oxide Synthase Structurally Resembles Cytochrome P-450 Reductase | 261 |
| | <i>David S. Bredt, Paul M. Hwang, Charles E. Glatt, Charles Lowenstein, Randall R. Reed, Solomon H. Snyder</i> | |
| 20 | Behavioral Abnormalities in Male Mice Lacking Neuronal Nitric Oxide Synthase | 277 |
| | <i>Randy J. Nelson, Gregory E. Demas, Paul L. Huang, Mark C. Fishman, Valina L. Dawson, Ted M. Dawson, Solomon H. Snyder</i> | |

Part VIII

D-AMINO ACIDS AS NEUROTRANSMITTERS

Commentary

| | |
|--|-----|
| Disruptive Science: Incongruent Findings Lead to Novel Insights Into How the Brain Works. | 291 |
| <i>Joseph T. Coyle</i> | |

| | | |
|-----------|--|-----|
| 21 | D-Aspartate Localizations Imply Neuronal and Neuroendocrine Roles | 297 |
| | <i>Michael J. Schell, Odelia B. Cooper, Solomon H. Snyder</i> | |
| 22 | Serine Racemase | |
| | A Glial Enzyme Synthesizing D-Serine to Regulate Glutamate-N-Methyl-D-Aspartate Neurotransmission. | 313 |
| | <i>Herman Wolosker, Seth Blackshaw, Solomon H. Snyder</i> | |
| 23 | Serine Racemase | |
| | Activation by Glutamate Neurotransmission via Glutamate Receptor Interacting Protein and Mediation of Neuronal Migration | 329 |
| | <i>Paul M. Kim, Hiroyuki Aizawa, Peter S. Kim, Alex S. Huang, Sasrutha R. Wickramasinghe, Amir H. Kashani, Roxanne K. Barrow, Richard L. Huganir, Anirvan Ghosh, Solomon H. Snyder</i> | |

Part IX

NEURAL MESSENGERS OF CELL LIFE AND DEATH

Commentary

| | |
|--------------------------------|-----|
| Beyond Neurotransmitters | 351 |
| <i>Samuel Barondes</i> | |

- 24** Cytochrome c Binds to Inositol 1,4,5-Trisphosphate Receptors, Amplifying Calcium-Dependent Apoptosis355
Darren Boehning, Randen L. Patterson, Leela Sedaghat, Natalia O. Glebova, Tomohiro Kurosaki, Solomon H. Snyder

- 25** Bilirubin Benefits
Cellular Protection by a Biliverdin Reductase Antioxidant Cycle383
Thomas W. Sedlak, Solomon H. Snyder

- 26** S-Nitrosylated GAPDH Initiates Apoptotic Cell Death by Nuclear Translocation Following Siah1 Binding.399
Makoto R. Hara, Nishant Agrawal, Sangwon F. Kim, Matthew B. Cascio, Masahiro Fujimuro, Yuji Ozeki, Masaaki Takahashi, Jaime H. Cheah, Stephanie K. Tankou, Lynda D. Hester, Christopher D. Ferris, S. Diane Hayward, Solomon H. Snyder, Akira Sawa

Part X

WHAT MAKES FOR CREATIVE DISCOVERY IN SCIENCE?

Commentary

| | |
|---|-----|
| What Creates Creative Science and Scientists? | 435 |
| <i>Nancy C. Andreasen</i> | |

- 27** The Audacity Principle in Science.439
Solomon H. Snyder

| | |
|-------------|-----|
| Index | 457 |
|-------------|-----|

About the Author

Solomon H. Snyder was born in Washington, D.C., in December 1938. Dr. Snyder received his undergraduate and medical training at Georgetown University (M.D. 1962); Research Associate training with Julius Axelrod at the National Institutes of Health (1963–1965); and psychiatric training at the Johns Hopkins Hospital (1965–1968). In 1966 he joined the faculty of the Johns Hopkins University School of Medicine (Assistant Professor of Pharmacology, 1966–1968; Associate Professor, Pharmacology/Psychiatry, 1968–1970; Professor, 1970). In 1980 he established the Department of Neuroscience and served as Director (1980–2006). He is presently Distinguished Service Professor of Neuroscience, Pharmacology, and Psychiatry.

Dr. Snyder is the recipient of numerous professional honors, including the Albert Lasker Award for Basic Biomedical Research (1978); the National Medal of Science (2005); the Albany Medical Center Prize (2007); Honorary Doctor of Science degrees from Northwestern University (1981), Georgetown University (1986), Ben-Gurion University (1990), Albany Medical College (1998), Technion University of Israel (2002), Mount Sinai Medical School (2004), and the University of Maryland (2006); the Wolf Foundation Prize in Medicine (1983); the Dickson Prize of the University of Pittsburgh (1983); the Bower Award of the Franklin Institute (1991); the Bristol-Myers Squibb Award for Distinguished Achievement in Neuroscience Research (1996); and the Gerard Prize of the Society for Neuroscience (2000). He is a member of the United States National Academy of Sciences and a Fellow of the American Academy of Arts and Sciences and the American Philosophical Society. He is the author of more than 1,000 journal articles and several books, including *Uses of Marijuana* (1971), *Madness and the Brain* (1974), *The Troubled Mind* (1976), *Biological Aspects of Abnormal Behavior* (1980), *Drugs and the Brain* (1986), and *Brainstorming* (1989).

Many advances in molecular neuroscience have followed from Dr. Snyder's identification of receptors for neurotransmitters and drugs and elucidation of the actions of psychotropic agents. He pioneered the labeling of receptors by reversible ligand binding in the identification of opiate receptors

and extended this technique to all the major neurotransmitter receptors in the brain. In characterizing each new group of receptors, he also elucidated actions of major neuroactive drugs. The isolation and subsequent cloning of receptor proteins stem from the ability to label, and thus monitor, receptors by these ligand binding techniques. The application of Dr. Snyder's techniques has enhanced the development of new agents in the pharmaceutical industry by enabling rapid screening of large numbers of candidate drugs. Dr. Snyder applied receptor techniques to elucidate intracellular messenger systems, including isolation of inositol 1,4,5-trisphosphate receptors and elucidation of inositol pyrophosphates as phosphorylating agents. He has established gases as a new class of neurotransmitters, beginning with his demonstrating the role of nitric oxide in mediating glutamate synaptic transmission and neurotoxicity. His isolation and molecular cloning of nitric oxide synthase led to major insights into the neurotransmitter functions of nitric oxide throughout the body. Subsequently, he established carbon monoxide as another gaseous transmitter and D-serine as a glial-derived endogenous ligand of glutamate–NMDA receptors. He has discovered novel mechanisms of cell death involving a nitric oxide–glyceraldehyde–3-phosphate dehydrogenase–Siah pathway as well as an IP_3 –cytochrome *c*–calcium cascade.

Foreword

Sol Snyder: Audacious Scientist, Leader of the Scientific Community, and Remarkable Human Being!

Eric R. Kandel

Sol Snyder, as you will learn from reading this collection of some of his best scientific papers, is an immensely creative person—one of the most creative in my generation of scientists. He has made numerous contributions to neuroscience and psychiatry that are not simply original, but often astonishingly so, because when they first appeared they were so audacious and unanticipated. Each of these discoveries has opened up a new area of understanding, and together they have revolutionized the modern study of the brain.

What I find particularly remarkable about Sol is that he is not only creative in science; he is creative in everything he does. There is a special “Sol” touch to all aspects of his life: an expression of an inner richness. Sol, to put it simply, is a savant of creativity. For example, he is an accomplished classical guitarist, and had he not become a scientist, he could have pursued a successful career as a professional musician. Overall, at every level, both public and private, Sol is a creative, social being, a friend of immeasurable generosity and thoughtfulness to his mentors, his students, and his colleagues.

Sol is very active in his community. He has brought his interest in music (along with exceptional organizational skills) to bear on the Baltimore Symphony Orchestra, on whose board he sits. In addition, he has served as the president of his synagogue, where he recruited its first woman cantor.

Sol was a student of Julius Axelrod, and during the time they worked together they established a wonderful mentoring relationship. Julie, as we all know him, has repeatedly said that one of the great satisfactions of his career was to have Sol as a student. And Sol, in turn, always emphasizes what a privilege it was to work for Julie. Both in word and action, Sol treated Julie as a father.

Sol is a supportive and entertaining husband, an involved father, and a doting grandfather. He recently described his trip to Disney World with his children and three grandchildren in one word: "Heaven!" When his first grandchild was born, every single lecture began with a picture of Abigail.

Sol's strong commitment to people extends naturally to his nurturing of his students, for whom he serves as a mentor par excellence. Sol's students rave not only about his creativity, but also about his thoughtfulness, his generosity, and his attention to their personal as well as professional lives. When meeting a student whom he has not seen for several years, he demands first off to see any baby pictures that might possibly have emerged. Reading letters of recommendation from Sol Snyder is like reading a nomination for the Lasker Award. In each of his students Sol brilliantly pinpoints the unique distinctive strengths so as to make the reader appreciate that he or she is dealing with a very special person—which is exactly how Sol feels about his students.

Sol is an extraordinary leader of the scientific community. At Johns Hopkins he formed the department of neuroscience, which he built into one of the major research groups in the country. In so doing he nurtured his faculty as he nurtured his laboratory and his family. As president of the Society for Neuroscience, in 1980 he launched our major journal, the *Journal of Neuroscience*, against considerable opposition, and established it as one of the premier journals in the field. Sol was also the first neurobiologist to become involved in biotechnology, and his first company, Nova, was a pioneer in the area. With part of the proceeds of this and his other ventures, Sol and his wife Elaine have contributed generously to Johns Hopkins, an institution Sol adores.

Sol has divided his collected papers, which form the body of this book, into ten sections, nine of which represent distinct scientific directions. Here, for more detailed discussion, I have combined some of these and emphasized others. I limit myself to four topics.

First, following up on his mentor Julius Axelrod's interest, Sol began to study neurotransmitter uptake as a mechanism for inactivating transmitters. By labeling receptors using reversible ligand binding, he succeeded in identifying opiate receptors, which are important for understanding pain perception. He then extended this method first to receptors for many of the neurotransmitters in the brain, including serotonin and dopamine, and then to subtypes of these receptors. The isolation of receptor proteins and their subsequent genetic cloning stem directly from the ability to label and thus monitor receptors by these ligand binding techniques. This advance represents a critical step toward understanding the fundamental molecular mechanisms whereby neurons communicate with one another.

Second, Sol has had a key role in the molecular characterization of the inositol 1,4,5-trisphosphate (IP_3) receptor, which is important for calcium

homeostasis. This experience put Sol in a position to explore the role of IP_3 as a potential target for lithium treatment in manic-depressive disorders.

Third, Sol has radically transformed conceptualizations of neurotransmission by establishing a gas, nitric oxide (NO), as a transmitter in the brain and by providing evidence that carbon monoxide may also be a neural mediator.

Finally, he has discovered that D-serine is the normal stimulus for the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor, which provides insight into how this receptor, important for learning and memory in the mammalian brain, is regulated.

Identification of Neurotransmitter Receptors by Labeling With Radioligands

It had long been known that drugs act via specific receptors. The possibility of labeling receptors with radioligands had been explored by numerous investigators, and some preliminary success was obtained in the late 1960s with [3H]atropine and the muscarinic receptor. However, the first successful labeling of neurotransmitter and drug receptors in the brain with reversible ligands began with Snyder's work.

In 1973 Sol Snyder and Candace Pert demonstrated opiate receptor binding in brain tissue. In their very first papers, their elucidation of receptor properties clarified fundamental questions about the actions of opiates. The relative affinities of opiates for binding sites were found to parallel pharmacologic activity, and opiate agonists and antagonists could be distinguished by the effects of sodium ion. The autoradiographic localization of opiate receptors that followed was critical for allowing us to understand how opiates exerted their pharmacologic actions, with receptors discretely localized to nuclei subserving functions influenced by opiates. These initial studies of opiate receptors provided powerful tools for screening new drugs in the pharmaceutical industry, and the discrimination of agonists and antagonists provided a first indication of how the recognition of a neurotransmitter or drug at its receptor might alter cellular events.

Sol next modified these techniques to develop a general strategy to label receptors for the major neurotransmitters in the brain. With Anne Young, he identified glycine receptors utilizing the antagonist [3H]strychnine. Glycine is an inhibitory neurotransmitter in the spinal cord and brain stem that hyperpolarizes motor neurons by increasing chloride conductance (the relative potencies of anions mimicking actions of chlorides being well established).

Labeling muscarinic cholinergic receptors with [3H]quinuclidinyl benzilate, Sol, working with Henry Yamamura, provided insights into important actions of psychoactive drugs. The potencies of antipsychotic neuroleptics

in blocking muscarinic receptors correlated inversely with their propensity for inducing extrapyramidal side effects. Influences of tricyclic antidepressants on these receptors provided a means of assessing the anticholinergic side effects of these drugs.

Sol, working with several students, next identified dopamine receptors in the brain, utilizing [^3H]dopamine and [^3H]haloperidol. He then showed that affinities of neuroleptics for dopamine receptors labeled by [^3H]haloperidol closely parallel their clinical and pharmacological activities, establishing that antipsychotic neuroleptic drugs act by blocking dopamine receptors. With Ian Creese and David Burt, Sol showed that the behavioral supersensitivity following destruction of the nigrostriatal dopamine pathway correlates closely with increased numbers of dopamine receptors. It is fair to say that the modern molecular era of transmitter receptors in the brain—an era that has revolutionized both the theory and practice of psychiatry—had its origin in Sol's lab.

The Function of Nitric Oxide in the Brain

Of the various roles of nitric oxide, its function as a major neurotransmitter was until quite recently the least explored. Sol's work in this area rapidly established a wholly novel concept of synaptic transmission—a gaseous neurotransmitter! Several of the principal insights into the functions and disposition of NO in the brain are attributable to Sol.

Following up on a key report by Garthwaite that brain cultures exposed to glutamate produce a substance that relaxes smooth muscle and behaves like NO, Sol with his Ph.D. student David Bredt sought and demonstrated the formation of NO from arginine. They next showed that glutamate, the major excitatory neurotransmitter in the brain, acting through the NMDA subtype of receptor, triples NO synthase (NOS) activity in a matter of a few seconds or shorter, which could mediate the simultaneous marked increase of cyclic guanosine monophosphate (cGMP) levels. NO formation causes the elevated cGMP, which Sol proved by showing that inhibitors of NOS block increased levels of cGMP formation.

Using antibodies to the purified enzyme, Sol, with Bredt and Ted Dawson, provided the first localization of NOS in the body and demonstrated selective localization to neurons in the brain and peripheral nervous system. The localization to the myenteric plexus of neurons in the gastrointestinal pathway suggested a role in peristalsis, which was demonstrated in numerous laboratories by showing that NOS inhibitors block relaxation of the intestines produced by physiological stimulation. Localization of NOS to penile autonomic neurons surrounding the blood vessels, whose engorgement with blood produces erection, enabled Sol and his colleagues to dem-

onstrate that NO is the physiological mediator of penile erections, as erections in intact animals produced by stimulation of the pelvic nerve are blocked by NOS inhibitors. These experiments establish NO as a neurotransmitter—which overturns classic concepts, since it is not a) stored in synaptic vesicles, b) released by exocytosis, c) bound to plasma membrane receptors, or d) inactivated by receptors or enzymatic hydrolysis.

In the brain, Sol found NOS neurons in discrete sites with a pattern of localization that did not correspond to that of any known neurotransmitters. He showed that the NOS neurons are identical to those that stain for NADPH diaphorase and that NOS activity accounts for diaphorase staining, since non-neuronal cells transfected with cloned NOS display staining for NOS and diaphorase in proportions similar to what occurs in neurons. Because diaphorase-staining neurons are known to be resistant to neuronal destruction following NMDA receptor stimulation in cultures as well as during vascular stroke and in Huntington's disease, Snyder, with Valina and Ted Dawson, postulated that stimulation of NOS by glutamate triggers the formation of NO by NOS neurons, with the NO released to kill adjacent cells.

Much of our understanding of the role of NO in biology has come from insights into properties of the NOS protein. Following NOS purification, Snyder and Brecht achieved the first molecular cloning of NOS, specifically of the brain form. The cloning studies enabled Snyder, in collaboration with Mark Fishman's laboratory, to generate mice lacking neuronal NOS. His demonstration that these animals have grossly enlarged stomachs with hypertrophy of the pyloric sphincter provides insights into the causation of human infantile pyloric stenosis, in which NOS neurons are also depleted in the pylorus.

Reasoning that NO may not be the only gaseous neurotransmitter, Snyder, with Ajay Verma, showed that carbon monoxide functions as a neuronal messenger.

Signal Transduction: IP₃ Receptors and Immunophilins

Snyder, with yet another group of students and postdoctoral fellows, utilized receptor strategies to clarify the actions of inositol trisphosphate, the mediator of the phosphoinositide cycle, which acts by releasing calcium. He identified receptor binding sites in the cerebellum, utilizing autoradiography to demonstrate remarkably high levels in discrete brain areas that greatly exceeded those in any other part of the body. Using biochemical methods, he purified the receptor to homogeneity. His reconstitution of the receptors into lipid vesicles in which the receptor protein mediates the ability of IP₃ to release calcium has established that the IP₃ receptor protein possesses both the

recognition site for IP_3 and the associated calcium ion channel. In the reconstituted receptor preparation, Snyder demonstrated quantal release of calcium, establishing how IP_3 can elicit exquisitely modulated pulses of calcium for intracellular signaling. Molecular cloning of this receptor by others, based on the isolation of the protein by Snyder's group, shows its close similarity to the ryanodine receptor of skeletal muscle, which also triggers calcium release. With Christopher Ross and Jacopo Meldolesi, Snyder showed the IP_3 receptor to be localized to elements of the endoplasmic reticulum including the nuclear membrane, providing a mechanism whereby the phosphoinositide system can enable neurotransmitter and hormonal messages to influence nuclear function. It is likely that the IP_3 receptor is the key intracellular recognition protein involved in communicating actions of intercellular messengers upon calcium within cells.

Sol's attention was suddenly directed to the immunophilins, and he went on to show them to be major signal-transducing proteins in the brain. Immunophilins were first identified as receptor proteins for immunosuppressant drugs. Previously, research on immunophilins had been restricted to lymphocytes and tissues of the immune system. With Joseph Steiner, Snyder demonstrated that the brain contains extremely high levels of the immunophilins, localized to discrete neuronal populations, with levels being 10–50 times greater than in immune tissues. He showed that drugs such as FK-506, by binding to immunophilins and inhibiting calcineurin phosphatase activity, can lead to accumulation of high levels of phosphorylated proteins in the brain. In this way, FK-506 enhances the phosphorylation of nitric oxide synthase. Since phosphorylation of NOS had been shown by Snyder to inhibit catalytic activity, treatment with FK-506 is equivalent to inhibiting NOS activity. Because NOS inhibition blocks glutamate neurotoxicity, Snyder and Dawson evaluated actions of immunosuppressants and showed that they block glutamate neurotoxicity in cultures.

Identification of D-Serine as the Coactivator of the NMDA Receptor

Most recently Sol has turned to the study of D-serine, which not only is an atypical isomer of the amino acid serine, but also occurs in glial cells rather than in neurons. Yet Sol has shown that despite these two improbable characteristics, D-serine acts as a candidate neurotransmitter. Snyder's immunohistochemical maps revealed that D-serine is present in a unique population of glia that surround nerve terminals selectively in a region of the brain that is rich in glutamate receptors of the subtype referred to as NMDA receptors. These receptors were thought to be coactivated by the amino acids glycine and glutamate. Sol found that D-serine is in fact the normal stimulus for the

glycine site of this receptor and that it is released from these glial cells by glutamate and then acts back on the NMDA receptor.

Any one of these four families of discoveries would be a full career for many scientists, but they represent only the core of Sol's remarkable fertile mind and his limitless creativity, energy, and enthusiasm. For these extraordinary contributions, Sol has been widely and importantly recognized. He is listed third in the Institute of Scientific Information list of the twenty-five most influential scientists in the last twenty years. He has received the highest honors America can bestow on its most gifted scientists. Among his many honors, Sol won the Lasker Award in 1978 and was elected to the National Academy of Sciences in 1980. In 2003 he received the National Medal of Science from President Bush.

But, as you will see when you read the papers that follow, none of these recognitions fully captures the range of contributions that have emerged from Sol's laboratory.

Eric R. Kandel, M.D., is University Professor at Columbia University, Fred Kavli Professor and Director at the Kavli Institute for Brain Sciences, and a Senior Investigator at the Howard Hughes Medical Institute. In 2000, his research concerning the molecular mechanisms of memory storage was recognized with the Nobel Prize in Physiology or Medicine.

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Introduction

Solomon H. Snyder

As a medical student at Georgetown University, 1958–1962, I had already formulated a career path: to become a psychiatrist and pursue a research career. A number of my classmates pointed out to me that for several years running, the magazine *Medical Economics*, their “bible,” had rated psychiatry as the lowest-earning specialty, usually tied with pediatrics, and that a career as a medical scientist was “off the charts” in the bottom direction. When I began psychiatry residency at Johns Hopkins in 1965, my fellow residents were not as dubious about research but nonetheless thought that I was a little peculiar for slipping away into the laboratory for so many hours a week. This volume is an attempt to clarify for a diverse audience, but especially for the psychiatric community, why fundamental research into the “messengers of the mind” is fun and important as much for clinicians as for researchers. The insights of modern neuroscience help make sense of the bewildering array of available psychotropic medications and also point toward novel, more effective, and safer agents.

The book comprises a series of key publications over the course of my roughly 40 years of brain research. To convey how all of this coheres, I will begin by recounting a little of my background, curiosities, aspirations, and motivations.

From boyhood, I wondered what the world was about. When I was a 7-year-old Hebrew day school student, I forever asked questions, none of which I recall, but which evidently were provocative. I was told that on one Sabbath several rabbis in the community formulated their sermons in response to a question posed by “a small boy at the Hebrew Academy.” At the same time I was curious about feeling states and motivations, perhaps influenced by my efforts to resolve bickering between my mother and father.

Calvin Coolidge High School, the public school I attended in Washington, D.C., was decidedly nonintellectual. We rarely received writing assignments, presumably because the English teachers were too lazy to grade long

essays. I benefited from this benign neglect by doing much reading on my own and writing short essays for myself. A gift of some philosophy books led to my reading works of Nietzsche and then Freud. However, the bulk of my free time in high school was devoted to playing classical guitar. When I was ten, my grandfather gave me one of his mandolins, which was much more fun than the piano and clarinet I had been studying since I was five years old. The one mandolin teacher my father could identify, Sophocles Papas, was primarily a teacher of classical guitar, the leading teacher in the country and Andrés Segovia's best friend. After a few years of mandolin lessons I switched to the guitar, which became my primary love. I practiced for hours every day and soon was performing in concert throughout the Washington area.

Senior year in high school presented a critical decision point. Mr. Papas could arrange for me to spend a summer in master classes with Segovia in Siena, Italy, after which I could enter a music conservatory and make the guitar my life. On the other hand, I wanted to "be like the other guys" who either were anticipating a career in engineering—the most rapidly expanding profession during those Eisenhower days of the military-industrial complex—or, like nice Jewish boys, were going to be premed college students. My mother, a decidedly atypical stage-mom Jewish mother, advocated Italy and the guitar. My father, a cryptanalyst at the National Security Agency, was enamored of science but wanted me to do whatever I preferred. Though I loved the guitar, I had a gnawing concern that a life in show business would be an itinerant, erratic venture, and, while intellectually adventurous, I was personally cautious. As for engineering, I hated math, slide rules, and mechanical drawing. In truth, I had little attraction to science of any sort. However, it occurred to me that if one could muddle through the sciences in college and medical school, a career as a psychiatrist, which could be conceptualized as akin to philosophy, might be attractive. I even had romantic notions of trying to understand "the mind." All rather silly, but somehow it all came to pass.

During my undergraduate years at Georgetown College, writing and philosophy became my passions. As a freshman I wrote on music for the school's literary magazine. I blossomed intellectually in junior year in a multi-credit philosophy course, composing essays on "being" and other abstractions linked to the writings of Saint Thomas Aquinas (Georgetown was a Jesuit institution). At the same time, I earned my way through school by running Mr. Papas' guitar shop on Saturdays and giving guitar lessons. One of my students, Donald Brown, had just graduated from medical school and was in the first class of Research Associates at the National Institutes of Health. He worked in the NIMH Laboratory of Clinical Science headed by Seymour Kety, then the doyen of biological psychiatry. During the summer before I was to begin medical school, Don needed a technician to work with him. I explained that I knew nothing of science and didn't particularly like

it, but he argued that it might be a nice opportunity for me to get a feel for the science of medicine, which might make the first year of medical school an easier undertaking.

At age 19 I fell in love again—with medical research, which was not at all like science in college but was as romantically creative as constructing a symphony or a painting. Don was studying the metabolism of the amino acid histidine by administering radiolabeled histidine to rodents, monkeys, and humans and fractionating urinary metabolites. His efforts were one ingredient in Dr. Kety's grand project to compare amino acid metabolism in schizophrenic individuals and matched control subjects. Kety was eager to verify or falsify the numerous published reports of abnormal biogenic amine metabolites in the urine of schizophrenics.

I arranged to spend all my summers and elective periods in medical school at the NIH. Though Don soon departed for a postdoctoral experience in France, I continued working in a semi-independent fashion. Don designed for me a project to identify and characterize a novel enzyme in the histidine metabolic pathway. Working with negligible supervision, I made every error imaginable, but after numerous false starts, I succeeded in characterizing the enzyme imidazolone propionic acid hydrolase. I wrote by myself a full-length manuscript, accepted without revision by the *Journal of Biological Chemistry*. Though I don't recommend the experience to aspiring scientists, I think that this initiation by fire facilitated my scientific development. With no formal mentorship, I secured advice by wandering the halls of the NIH asking questions of all the biochemists I knew—many of whom were my guitar students.

At the same time, I became enmeshed in the psychology of schizophrenia. My sister Elaine's husband, Irving Taylor, had published award-winning research developing simple paper-and-pencil tests to evaluate Gestalt principles such as "closing gaps" in emotionally relevant figures—perceptual closure. He showed that subjects who are politically right-wing or left-wing "close gaps" much more than moderates. I wondered how schizophrenic patients would perform. Don suggested that I ask Dr. Kety for permission to administer the tests to NIH patients, leading to my first "seminar" to the Laboratory of Clinical Science staff, an audience of almost 100—a daunting experience for a 20-year-old. All went well, and Kety assigned David Rosenthal, head of the Section on Psychology, as my mentor. For the rest of my medical school career, besides working in the biochemistry lab, I administered the Gestalt tests to schizophrenic subjects, first at the NIH and then to a larger patient population at St. Elizabeths Hospital. We made a number of interesting observations. Chronic schizophrenics were less likely to manifest perceptual closure than normal subjects, while acute paranoid schizophrenics displayed greater closure. Also, schizophrenics were notably more

variable in their performance than healthy subjects in interesting ways, shedding light on the well-known variability of schizophrenic performance in various test systems. There ensued publications in the *Journal of Abnormal Psychology* and *Archives of General Psychiatry*, several of which David Rosenthal wrote but one of which I wrote on my own.

I was hooked on research but still wanted to be a psychiatrist. Moreover, the feared “doctors draft” loomed. I considered applying to the NIH as a Clinical Associate in Psychiatry after two years of residency, with the NIH experience comprising both my military service and the last year of residency. However, Elaine Borko and I had decided to wed. To complete college she was required to do “practice teaching” in Washington, D.C., so I decided to seek a research position at the NIH for the year after internship. All the Research Associate positions were already filled in a matching plan. After several fruitless explorations, I visited the lab directly across the hall from the one in which I had worked throughout medical school, the laboratory of Julius Axelrod. I knew Julie from frequent encounters in the hallways and because he had given me some advice on histidine conversion into histamine, an area of his research. Julie commented, “Sol, there are only a dozen Research Associate positions in the whole NIH, with thousands of applicants. Research Associates are all valedictorians from Harvard or Yale—and you went to Georgetown. However, the fellow who matched with me just dropped out, so you can have a position.” That ten-minute interview changed my life.

Elaine and I were married June 10, 1962, and drove off that day to San Francisco, where I interned at the Kaiser Foundation Hospital. One year later I commenced an extraordinary two years with Julie.

I joined Julie in 1963, only eight years after he obtained his Ph.D. at the age of 42 following nine years as a technician, during which time he had discovered the analgesic acetaminophen (Tylenol) and the cytochrome P450 drug-metabolizing enzyme system. In his post-Ph.D. eight years he had discovered catechol-O-methyltransferase (COMT) and the entire metabolic pathway of catecholamines, identified reuptake as a major mechanism for inactivation of norepinephrine, and shown that cocaine and antidepressant drugs act by blocking this uptake system. In a tiny laboratory with no more than two to four trainees at a time, he revolutionized the field of drug metabolism and molecular neuropharmacology and trained a number of world leaders. Many people wonder what magic spawned Julie’s scientific creativity and his talents as a mentor. I’ll try to provide some insights by describing my own experience.

Julie did not believe in a laissez-faire system in which students flounder as did I in my medical student days. He felt that positive reinforcement was critical and so would start each trainee with a straightforward, circum-

scribed project, one that had a high probability of success but just might lead to a major breakthrough. My first project was to investigate the disposition of radiolabeled histamine in rodents. Julie's reasoning was simple. His discovery of reuptake inactivation of norepinephrine had come from a similar administration of radiolabeled norepinephrine to rats, after which he observed a high concentration of the label in organs with rich sympathetic innervation. Since I had some experience with histidine and histamine, I would have something to offer in addition to learning new experimental strategies.

Julie would gradually wean students, allowing each of us more and more latitude as we showed we could think for ourselves. He was most delighted when we would hatch our own ideas, do pilot experiments, and then surprise him with our findings. Julie also emphasized the importance of developing simple, sensitive, and specific means of measuring enzymes or small molecules, frequently admonishing, "The biggest advances in science always come from new methodology that permits measuring things simply and rapidly." Thus, when Julie read an article mentioning that heating serotonin together with the ninhydrin reagent used to stain proteins resulted in a highly fluorescent product, he suggested that I develop this into a new methodology for detecting serotonin. Within a few weeks I had devised a method that was about 10 times more sensitive than the conventional one. At that time, the hottest thing in the laboratory was research on the pineal gland hormone melatonin, which is formed from serotonin. Wilbur Quay had published a study demonstrating that serotonin content of the pineal gland showed a circadian rhythm, with levels at noon roughly 10 times values at midnight. Since the pineal gland weighs only about 1 milligram, Quay consumed several hundred rats in a single heroic experiment identifying this cycle. Utilizing the new ninhydrin technique, I could measure serotonin in two rat pineal glands, permitting many experiments. Thus, we were able to show that the serotonin rhythm is endogenous, persisting in constant darkness. Light exposure blocked the nocturnal decline in serotonin, enabling us to lesion the visual system and map the pathway of light to the pineal gland.

After two years with Julie I came to Johns Hopkins for psychiatry residency. I secured an arrangement whereby the first year was full-time residency but during the second year I would be both a resident and an assistant professor of pharmacology with a laboratory and a technician. One important benefit was that my salary as a faculty member vastly exceeded the \$250 per month that psychiatry residents were paid. Accordingly, after four years of marriage Elaine was able to stop working and we could begin a family. Another benefit was a head start on my research career. I was assigned to lecture sophomore medical students on psychopharmacology. Two became my first trainees—Joseph Coyle, now a professor of psychiatry at Harvard, and Alan

Green, now Chair of Psychiatry at Dartmouth—and another became my first Ph.D. student, Michael Kuhar, now a professor of pharmacology at Emory.

Our early projects dealt with neurotransmitter uptake as a mode for synaptic inactivation of neurotransmitters. Most of Julie's studies employed intravenous injections or intraventricular injections of radiolabeled norepinephrine, an approach that precluded analysis of the transport process. Utilizing isolated perfused rat hearts, Leslie Iversen had shown that norepinephrine transport obeys Michaelis-Menten kinetics much like an enzyme, but this was a cumbersome approach that used large numbers of rats. Brain slices worked poorly because of variations in surface area. In landmark studies, Victor Whittaker homogenized brains very gently in sucrose solutions, whereupon nerve endings literally pinched off and sealed up to form isolated nerve ending particles, which he dubbed synaptosomes. Some workers had attempted to monitor norepinephrine uptake into synaptosomes in sucrose solutions but failed, presumably because transport processes are typically coupled to the sodium pump so that no transport can take place in pure sucrose. However, exposure to a sodium-containing salt solution would disrupt synaptosomes. Coyle devised a deceptively simple approach, preparing crude synaptosomes in sucrose solutions and then adding a sodium phosphate buffer that would not harm the nerve endings, now protected by the coating of sucrose. We soon differentiated discrete transporters in dopamine and norepinephrine neurons. We then discovered that conventional antiparkinsonian drugs, such as benztropine (Cogentin), presumed to act as anticholinergics, were potent and selective inhibitors of dopamine transport at brain concentrations associated with therapeutic doses.

This uptake technology helped resolve the status of amino acids as neurotransmitters. In the early 1950s Eugene Roberts had discovered the amino acid γ -aminobutyric acid (GABA) as a brain-specific molecule, subsequently established as an inhibitory neurotransmitter. In the 1960s several investigators found that conventional amino acids such as glycine, glutamate, and aspartate could potentially excite or inhibit neurons, suggesting neurotransmitter roles. However, just because applying a substance changes neuronal firing, this does not mean that the substance is normally formed and released to act on the target neurons. If these amino acids are neurotransmitters, their disposition must somehow differ from those of other amino acids. The brain must be able to discriminate the neurotransmitter pool of the amino acids from the presumably larger pools devoted to general metabolism as well as protein synthesis. Evidence was accumulating that reuptake might be a universal mode of synaptic inactivation. Hence, we speculated that if an amino acid were a neurotransmitter, it should possess a high-affinity, sodium-requiring transporter that could be differentiated from the more generalized low-affinity transport whereby amino acids enter

all cells of the body. Utilizing our synaptosomal preparations, Bill Logan and Jim Bennett showed that glycine, glutamate, and aspartate are subject to high-affinity, sodium-requiring transport not evident with other amino acids. Interestingly, neurophysiologists had shown that glycine mimics the actions of the natural inhibitory neurotransmitter in the spinal cord but not in higher brain centers, a finding that meshed with our observations of high-affinity transport for glycine in the spinal cord but not the cerebral cortex.

In 1969 I gave a lecture at the Lilly drug company describing our “high-throughput” approach to monitoring neurotransmitter uptake. I recall well that two Lilly neuropharmacologists, Ray Fuller and David Wong, peppered me with questions. More than 20 years later I read a book by Peter Kramer, *Listening to Prozac*, and learned that following my visit to Lilly, Wong and Fuller devised a strategy for screening large numbers of chemicals for differential inhibition of serotonin or norepinephrine transport, leading to the novel serotonin selective reuptake inhibitor fluoxetine (Prozac). Just as Julie had never patented his conceptualization of acetaminophen as a novel analgesic, so we never patented the synaptosomal uptake system as a method for drug discovery.

Studies of neurotransmitter transport and amino acids as neurotransmitters occupied us through the late 1960s and early 1970s. This brings us to the era of the papers presented in this volume, the first being on the discovery and characterization of opiate receptors. After Richard Nixon declared “war on heroin,” he appointed the psychiatrist Jerome Jaffe as his anti-drug abuse czar, with vast resources of manpower and money to attack the widespread use of heroin among our soldiers in Vietnam as well as addicts on the streets of major American cities. Arnold Mandell, then Chair of Psychiatry at the University of California at San Diego, and I importuned our friend Jerry to designate some funds for basic research centers on drug abuse. This led to the funding by William (Biff) Bunney, Director of the NIMH Center for Drug Abuse (the precursor of the National Institute on Drug Abuse), of a few drug abuse research centers, including one at Johns Hopkins.

Saddled with the responsibility of doing something in an area in which I had no previous experience, I read the literature on opiates and concluded that the dearth of fundamental insights into opiate action stemmed from the failure to identify the initial target of opiates, a presumed “opiate receptor.” The distinguished pharmacologist Avram Goldstein had monitored the binding of radiolabeled opiates to brain membranes but had found only negligible levels associated with specific sites. Pedro Cuatrecasas, whose laboratory was adjacent to mine, had pioneered insulin receptor binding technology, and we had collaborated in identifying receptors for nerve growth factor. This familiarity with the “tricks of the trade” in receptor research told me why previous efforts had missed the mark—and permitted the identifi-

cation of opiate receptors. We used opiates of high specific radioactivity, mixing them with crude brain membranes, which were washed extensively to remove nonspecific binding but rapidly enough to preserve receptor interactions. What these studies taught us about how opiates relieve pain and cause euphoria and how to differentiate agonists and antagonists is the subject of the papers presented in Part 1 of the book.

Man was not born with morphine in his brain. Soon after the identification of opiate receptors, numerous laboratories sought endogenous morphine-like substances in the brain. We made use of our receptor binding technology, seeking something in the brain that would compete with the binding of radioactive opiates to the opiate receptor. To ensure that we were not dealing with nonspecific inhibition of receptor binding, we looked for a substance whose concentrations varied throughout the brain in proportion to the density of opiate receptors. Independently, in Aberdeen, Scotland, Hans Kosterlitz and John Hughes took advantage of the ability of opiates to inhibit electrically induced contractions of smooth muscle preparations. They found substances whose actions were blocked by the opiate antagonist naloxone, ensuring that they were not dealing with nonspecific effects. The Scottish team were first to obtain the chemical structures of the enkephalins, the first opioid peptide neurotransmitters (endorphins), a couple of months before us. Part 2 of the book reprises characterization of the enkephalins.

Successful labeling of receptors by binding techniques requires a radio-labeled ligand that has high affinity for receptors and manifests very few nonspecific interactions with tissue membranes. By seeking just the right ligands, within a few years we successfully labeled receptors for the major neurotransmitters in the brain. The set of papers composing Part 3 of this book deals with dopamine receptors, which are important to psychiatry because therapeutic actions of antischizophrenic neuroleptic drugs reflect their blockade. The close correlation of clinical and pharmacological potencies for neuroleptics established dopamine receptors as the therapeutic target.

Besides elucidating therapeutic actions of drugs, receptor actions account for side effects. Presently pharmaceutical companies screen hundreds of thousands of chemicals for actions both on receptors mediating therapeutic effects and on those responsible for side effects. Among neuroleptics, we found that potent anticholinergics such as clozapine have fewer extrapyramidal side effects than agents such as haloperidol, which have negligible anticholinergic potency. Among both neuroleptics and tricyclic antidepressants, blockade of alpha-noradrenergic receptors is associated with sedative-hypotensive actions. Drug actions on receptors are the topic of Part 4.

Receptor subtypes permitting selective actions of neurotransmitters in different organs have been evident for many years, exemplified by muscarinic and nicotinic cholinergic receptors as well as alpha- and beta-adrenergic

receptors. With drugs as radioligands for receptors, we observed differential binding properties that reflected different receptor subtypes. It was possible to discriminate at least two serotonin receptors, designated 5-HT₁ and 5-HT₂, which are associated with separate physiological functions. The advent of gene cloning has led to identification of at least a dozen serotonin receptors. Drugs selective for various subtypes have major therapeutic importance, such as the triptan drugs for treating migraine and 5-HT₃-selective drugs like ondansetron (Zofran) for relieving nausea and vomiting associated with cancer chemotherapy. Serotonin receptor subtypes and their modulation by antidepressants are the subject of the next section of papers (Part 5).

Initial binding studies dealt only with the recognition site of neurotransmitter receptors. Somehow this recognition information is transduced into second messenger systems, of which cyclic adenosine monophosphate (cAMP) was the first generally recognized second messenger. In the mid-1980s the work of Michael Berridge and others identified inositol 1,4,5-trisphosphate (IP₃) as a novel second messenger that releases intracellular calcium following activation of hormone and neurotransmitter receptors. Calcium is released by IP₃ from small vesicles of the endoplasmic reticulum inside cells, suggesting the existence of specific IP₃ receptors. Soon we successfully labeled IP₃ receptors. In those days, receptor research employed intact membranes because the science of solubilizing and purifying membrane proteins was quite primitive. Through patience and luck, we solubilized and purified IP₃ receptors. With isolated IP₃ receptor protein, we could ask whether the receptor contained only the IP₃ recognition site or whether, instead, the associated calcium ion channel was an intrinsic part of the same protein molecule. We reconstituted purified IP₃ receptors into lipid vesicles that we loaded with radioactive calcium, and in this way we were able to show that IP₃ selectively released calcium from the receptors inside the vesicles, establishing that the calcium ion channel is indeed part of the IP₃ receptor.

Elucidating the mechanism of therapeutic action is easy with some drugs and notoriously difficult for others. Best are drugs that act at very low concentrations, hence are highly selective, and for which a homologous series of agents exists so that one can correlate potency at a candidate target with therapeutic effectiveness. Neuroleptics are ideal for such studies. Lithium, on the other hand, poses major challenges since its therapeutic blood concentration is millimolar, reflecting a potency about a million times less than drugs such as haloperidol. Moreover, there are no homologous series of drugs to compare with lithium—sodium and potassium being rather poor candidates. Berridge first proposed that the perplexing ability of lithium to decrease manic highs as well as alleviate depression might stem from a “nor-

malizing” action on the phosphoinositide cycle of metabolic events associated with IP_3 . Using combined biochemical and neurophysiologic strategies, we obtained evidence supporting this possibility and pinning down relevant molecular mechanisms. These studies, as well as work on IP_3 receptors, make up a section of papers (Part 6).

The first neurotransmitters were all biogenic amines such as norepinephrine, dopamine, acetylcholine, and serotonin. In the 1960s amino acids were proposed, and are now appreciated, as the quantitatively predominant neurotransmitters in the brain. The 1970s were the decade of neuropeptides, with major interest sparked by the discovery of enkephalins/endorphins. By the 1990s the concatenation of amines, amino acids, and peptides seemed more than enough to fill the brain with neurotransmitters, though an important principle was emerging, namely that every neuron possesses at least two and as many as four or five neurotransmitters.

In the late 1980s nitric oxide (NO) was identified as a major regulator of blood vessel function and of the killing by macrophages of tumor cells and bacteria. We wondered whether NO might serve some function in the brain and were able to show that it mediates the ability of glutamate to enhance cGMP levels. Being a gas, NO cannot be stored in synaptic vesicles and released at leisure. Instead, presumably some enzyme must resynthesize NO every time a molecule is released upon nerve firing. Identifying such an enzyme would be critical for advancing the field. Several groups had attempted to find the presumed NO synthase, which would convert arginine into NO and citrulline. All efforts had failed because the enzyme appeared to be incredibly labile; we discovered, however, that the enzyme was not labile but that during purification, investigators had removed the calcium binding protein calmodulin, which is a critical component of the enzyme complex. This discovery explained how neuronal firing can activate NO formation, since neuronal depolarization is associated with calcium influx into nerve terminals. Once we purified and then cloned the gene for neuronal NO synthase, we were able to develop mice with genetic deletion of the enzyme. The extraordinary hypersexuality and intensely aggressive behavior of these mice provided insights into the normal role of NO as a neurotransmitter. Papers on the NO story compose Part 7 of the book.

NO overturned many “rules” of neurotransmission, such as a requirement for storage in synaptic vesicles, release by exocytosis, and action upon specific membrane receptors. Instead, NO merely diffuses into adjacent cells and stimulates the formation of cGMP or nitrosylates selected proteins. Our studies of D-amino acids as neurotransmitters confound even more dogma. Biology is stereospecific with only D-sugars and L-amino acids. A group of Japanese investigators had discovered that while, for the most part, the body uses only L-amino acids, the brain contains substantial levels of D-serine and

D-aspartate, with D-serine levels being about a third those of L-serine. We showed that D-serine is the endogenous ligand for the so-called glycine site of the glutamate–N-methyl-D-aspartate (NMDA) receptor. Glutamate exerts its excitatory actions via several different receptor subtypes, of which the NMDA receptor is particularly prominent, being critically involved in the long-term potentiation that gives rise to learning and memory. For years it was known that to activate NMDA receptors, stimulation of the glutamate binding site is insufficient. A second site must also be engaged, and glycine worked well at this site. Why should there be two neurotransmitters for a single receptor? Researchers speculated that accidental overactivation of NMDA receptors by dietary glutamate might be dangerous, so that nature designed “a lock that requires two keys.” However, glycine, like glutamate, is abundant in diets. We showed that selective degradation of D-serine but not glycine abolishes glutamate-NMDA neurotransmission. We cloned an enzyme, serine racemase, which converts L-serine to D-serine. We also demonstrated an important role of D-serine in regulating neuronal migration during the development of the nervous system. The series of papers that makes up Part 8 describes these features of D-serine as well as D-aspartate, the only other D-amino acid in nervous tissue.

Apoptosis, a form of programmed cell death, is increasingly appreciated as a principal means whereby half the neurons generated during embryonic life drop out before birth. Programmed cell death also appears critical for the pathophysiology of stroke and various neurodegenerative diseases as well as the death of myocardial and other peripheral tissues. In many disorders cell death is typically initiated by reactive oxygen species that are formed as a by-product of normal mitochondrial metabolism. To cope with these substances, the body has elaborated endogenous antioxidant systems, of which the best known is the tripeptide glutathione. Recently we discovered that bilirubin, generally regarded as a toxic by-product of heme metabolism, is an important physiologic antioxidant. We have also elucidated two novel pathways of apoptotic cell death. In one of these, release of cytochrome *c*, known to initiate apoptosis, binds to IP₃ receptors, amplifying the release of calcium. In another, NO chemically modifies by nitrosylation the house-keeping glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to its nuclear translocation and cell death. These stories constitute the penultimate section of the volume.

The final section comprises a single paper, “The Audacity Principle in Science.” In it, I speculate about factors that make for creativity and efficacy in scientific discovery.

Creativity and mentoring are perhaps the guiding themes of my research career. Even in my days with Julie Axelrod, I was far more enthusiastic about an idea that was my own as opposed to a suggestion of Julie’s, even though

his ideas were usually better than mine. Today I remain obsessed with the need to “think thoughts no one else has thought before.” Students can see my eyelids falling as they describe experiments that are frankly boring, while I perk up, no matter how little sleep I had the preceding night, when they report on something decidedly novel.

An urge to find something new accounts for the diversity of problems our laboratory has attacked over the years. I recall well the initial period following the identification of opiate receptors and their endogenous neurotransmitters, the enkephalins. My senior advisors admonished, “Sol, this opiate receptor protein is clearly of such great importance with all sorts of ramifications that you should set everyone in your lab to work on it and stick with it for the next twenty years.” Despite the ravishing early days of research on the opiate receptors, after a few years I could see a point of diminishing returns. By contrast, receptors for all of the other major neurotransmitters beckoned, affording diverse questions: How do neuroleptics act? What mediates side effects of antidepressants? The reversible ligand-binding strategy that worked for opiate receptors could be readily transferred to others if one could come up with the ideal, high-affinity radiolabeled ligand. Once the principal neurotransmitter receptors had been characterized, we were able to use similar techniques to address other high-affinity binding proteins such as odorant-binding proteins; second messenger targets such as the IP_3 receptor; and receptors for the immunosuppressants that stimulate nerve growth, the immunophilins.

I continue to be restless intellectually. Currently our laboratory is heavily involved in studies of mechanisms of neuronal death and cytoprotective systems. It would be heartwarming to find the specific molecular underpinnings of major mental disorders and neurodegenerative conditions such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease. However, even knowing the exact causes may not provide curative treatments—as is evident for Huntington’s disease, whose genetic origins have been evident for over a decade but with no hints for curative therapy. By contrast, an understanding of the mechanisms of neuronal death may lead to neuroprotective drugs for a wide range of conditions. Blocking the excitatory actions of glutamate has already afforded potential antistroke drugs. Recently we uncovered a cell death—signaling cascade that involves nitric oxide. Diverse cell stressors all trigger formation of NO, which modifies the housekeeping glycolytic enzyme GAPDH. This nitrosylation modification abolishes the enzyme’s catalytic activity but confers on it the ability to bind to a protein called Siah, which transports GAPDH to the nucleus, where it interacts with other proteins that kill the cell. The monoamine oxidase inhibitor deprenyl, long used to treat Parkinson’s disease by elevating brain dopamine levels, is neuroprotective in many animal models and appears to delay the loss of

dopamine neurons in Parkinson's disease. We discovered that deprenyl at very low concentrations blocks the binding of GAPDH to Siah. Other, more selective drugs exerting this effect might be useful in treating neurodegenerative conditions.

All of the above is about “me, me, me”—but this isn't how research is done, at least not in my lab. Advising, encouraging, and brainstorming together with my students is every bit as important as making creative discoveries. Because I am a klutz in the laboratory, there would be no discoveries if they depended on my two hands. Moreover, all of the “original” ideas flow from an interplay of my students and myself trading half-baked ideas back and forth. My greatest joy is seeing the original conceptualization emerge from the student rather than from me. I suspect that this mentoring process is by no means unique to myself but characterizes all apprenticeship in biomedical research.

That's what it's all about: Work with young talents in a warm, intellectually stimulating interplay, think about the big questions in the brain and psychiatry, and go for broke.

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Part I

DISCOVERY AND CHARACTERIZATION OF THE OPIATE RECEPTOR

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COMMENTARY

Radioligand Binding Methodologies: New Inventions, New Directions

Eric J. Nestler

My first foray into the world of neuropharmacology was in January 1975, when, as a junior at Yale College, I took a course—Introduction to Neuropharmacology—at the nearby medical school. The course used the brand new second edition of *The Biochemical Basis of Neuropharmacology* by Cooper, Bloom, and Roth (1) as its textbook. It is very interesting to think back to those still early days of the field and consider how remarkably things have changed in 30 years.

Back in 1975, the course and the textbook focused almost exclusively on monoamine neurotransmitters and acetylcholine, with only brief mention of amino acids. There was virtually no reference to peptide neurotransmitters. A great deal was already known about the metabolism of monoamines and acetylcholine, but very little about their receptors. Numerous receptor subtypes were already defined, but mainly as theoretical and functional constructs, not as true biochemical entities. Thus, all receptor classifications were based solely on the pharmacological activity of large numbers of receptor agonists and antagonists in organ- or cell-based or even whole animal preparations. Lecturers in the course made passing mention of a few recent papers by Sol Snyder and others on a new method of receptor binding, but no information on the biochemical and molecular properties of receptors was available. Only cyclic nucleotide second messengers were known, Ca^{2+} was just beginning to be thought of as an additional second messenger, and our

understanding of the molecular pathways through which receptor activation recruits these second messengers was very limited; for example, G proteins had not yet been discovered. Today, it is easy to be frustrated by the perception of slow progress in neuropharmacology and to focus on important areas still awaiting discovery (e.g., genes that confer risk for mental illness), but it is very striking and very heartening to appreciate the transformation in neuropharmacology that has taken place over the past three decades.

The early studies by Candace Pert, Gavril Pasternak, and Sol Snyder (2, 3), reproduced in this volume as Chapters 1 and 2, played a key role in paving the way for many of these momentous advances. The seminal technical innovation offered by these studies was the introduction of radioligand binding assays to characterize neurotransmitter receptors. An earlier publication by Avram Goldstein and colleagues in 1971 (4) had reported stereoselective binding of a radiolabeled opiate ligand to brain extracts, but the signal, relative to background noise, was minuscule (~2% of total binding). In 1973, by use of a radioligand of much higher specific activity, and by more thorough and rapid washing of the extracts, Pert and Snyder (2) were able to dramatically increase the signal-to-noise ratio, which made it possible to use this binding to characterize the newly discovered opioid receptor. They demonstrated for the first time the localization of the opioid receptor to nervous tissue and its enrichment in particular brain regions, a subject further characterized in a related publication in *Nature* that same year (5). The discovery made it possible to begin characterizing the opioid receptor at a biochemical level—for example, demonstrating its proteinaceous nature (6; see Chapter 3). Pert and Snyder (2) also were able to characterize the pharmacology of the opioid receptor by studying the ability of a relatively large number of agonists and antagonists to interfere with radioligand binding in brain extracts. Indeed, the stereoselectivity of the binding, and the fact that the relative affinities of various opiate ligands to the binding site matched their pharmacological potency, supported the notion that the binding site was a true (physiological) opioid receptor. Several other groups helped validate this new approach to studying receptors by independently demonstrating opioid receptor binding in nervous tissue, as summarized by Pasternak and Snyder (7) in an excellent historical review of these initial discoveries.

Still in 1973, the Snyder laboratory followed with a second seminal publication, which provided the first means of distinguishing between a receptor agonist and antagonist in an *in vitro* (cell-free) assay (3). They discovered that Na⁺ ions reduce the binding of radiolabeled agonists to the opioid receptor in brain extracts, whereas Na⁺ increases binding of radiolabeled antagonists. They then showed that the ability of non-radioactively labeled ligands to interfere with [³H]naloxone (an antagonist)—in the presence or absence of Na⁺—easily differentiated functional agonists from antagonists.

These technical advances rapidly heralded a new era of neuropharmacology, which led to the identification and characterization of one neurotransmitter receptor after another—by the Snyder laboratory and several other groups—through the mid-1970s (7). By the time of the third edition of Cooper, Bloom, and Roth, published in 1978, neurotransmitter receptors were understood to be biochemical entities and their physicochemical properties were being revealed. Very significantly, the advent of radioligand binding assays revolutionized the drug discovery process. As the first truly high-throughput assay available to the industry, radioligand binding became widely used and made it possible for pharmaceutical companies to rapidly and efficiently screen large numbers of chemicals as agonists or antagonists of a receptor of interest. Radioligand binding was also extended by Snyder and other investigators to other, non-receptor proteins—including plasma membrane transporters and even intracellular signaling proteins, as just two examples (7, 8). Radioligand binding was similarly expanded to the method of receptor autoradiography, which made it possible to study receptor sites in brain sections, thereby providing ever greater anatomical information concerning receptor distribution in the nervous system. The ultimate extension of radioligand binding was the invention of ways to image receptors in the brains of living animals and humans through such methodologies as positron emission tomography and single-photon emission computed tomography. The field is still reaping the benefits and promise of these latest applications of radioligand binding in terms of improved diagnosis of neuropsychiatric diseases in humans.

Beyond the extraordinary technical leaps made possible by the invention of radioligand binding are the many important conceptual advances also facilitated by these early studies by Snyder and coworkers. Their early experiments, which revealed fundamental functional differences between the actions of agonists and antagonists at the opioid receptor, led directly to discoveries of how different classes of ligands bind receptors and how that binding is translated into biological effects—for example, through interactions with G proteins. Additionally, such studies provided the foundation for research that established rational drug design of receptor agonists and antagonists and elucidated the structure of G protein-coupled receptors.

Furthermore, the presence of an opioid receptor in brain, distinct from monoamine and acetylcholine receptors, suggested very clearly that the brain must contain endogenous ligands for that receptor. Indeed, the discovery of endogenous opioid peptides by Snyder and others followed rapidly from the Snyder laboratory's initial studies of opioid receptors, as described in the next section of this volume. The appreciation that endogenous opioid neurotransmitters are peptides subsequently triggered an explosive period in neuropharmacology research that continues to the present day, during

which an increasingly large number of peptides that serve neurotransmitter functions in the brain have been discovered.

Finally, the discovery and initial characterization of the opioid receptor made it possible for the first time to study the detailed mechanisms by which opiate drugs, such as morphine, change the brain to cause addiction. This work provided the foundation for much of my own laboratory's research on the molecular mechanisms of addiction, which dates back to the mid-1980s. Our work, focusing on post-receptor mechanisms of opiate addiction, was a direct corollary of an early observation made by Pert and colleagues in 1973 (3). Those investigators showed that brain levels of the opioid receptor itself are not altered during the development of opiate dependence—thus pointing the field toward post-receptor mechanisms.

On a more personal note, I would like to congratulate Sol for this wonderful book, which tracks the enormous discoveries made by his laboratory dating back to the early investigations of opioid receptor binding in brain. I also want to congratulate Sol for his extraordinary mentoring: the large number of national leaders today who began their careers in the Snyder laboratory at Johns Hopkins is not merely impressive, it is staggering! But Sol's mentorship in the field goes well beyond his own laboratory, given the many other individuals whose careers he has promoted and nurtured over the years. This has been particularly true for physician-scientists, for whom Sol has been an exemplary role model. I count myself squarely in this category and thank Sol for all the inspiration, support, and advice he's generously given me ever since I entered our exciting field years ago.

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CHAPTER 1

Opiate Receptor

Demonstration in Nervous Tissue

Candace B. Pert
Solomon H. Snyder

Pharmacological evidence for the existence of a specific opiate receptor is compelling, but heretofore it has not been directly demonstrated biochemically. We report here a direct demonstration of opiate receptor binding, its localization in nervous tissue, and a close parallel between the pharmacologic potency of opiates and their affinity for receptor binding.

Rats, guinea pigs, and mice were killed by cervical dislocation. Their brains were homogenized in six volumes of 0.05 M Tris-HCl buffer, pH 7.4, and diluted to 18 times the original volume of the same buffer. Portions (2 mL) were incubated for 5 minutes at 35°C with various drugs, and the incubation was continued for 15 minutes after the addition of (–)-[³H]naloxone (5×10^{-9} M final concentration). Samples were cooled to 4°C, filtered through Whatman glass fiber circles (GF-B), and the filters were washed under vacuum with two 8-mL portions of ice-cold Tris buffer. The filters were extracted by shaking with 1 mL of 10% sodium dodecyl sulfate in counting vials for 30 minutes, and radioactivity was determined by liquid scintillation spectrometry after the addition of 12 mL of PCS counting fluid (phase combining system; Amersham/Searle). Other tissues were assayed for opiate

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receptor binding in homogenates or minces by filtering (Tables 1-1 and 1-2). All determinations of binding capacity were performed under conditions in which binding was linear with tissue concentration.

(-)-Naloxone was labeled by tritium exchange at the New England Nuclear Corporation as follows. A portion (50 mg) was dissolved in 0.3 mL of trifluoroacetic acid with 50 mg of 5% Rh on Al_2O_3 to which was added 25 curies of $[\text{}^3\text{H}]$ water; the mixture was then incubated for 18 hours at 80°C . In our laboratory, a 70-mc portion of the $[\text{}^3\text{H}]$ naloxone was evaporated to dryness twice, purified by thin-layer chromatography (*n*-butanol, glacial acetic acid, H_2O , 4:1:2), and its purity was verified in three other thin-layer systems. The specific activity of $[\text{}^3\text{H}]$ naloxone was 6.1 c/mmole as determined by comparison with the ultraviolet absorption of standard solutions of naloxone.

The analgesic activity of opiates is highly stereospecific, with almost all activity residing in those isomers with a configuration analogous to that of $\text{D}(-)$ -morphine. Accordingly, on the assumption that analgesic potency parallels affinity for binding to the opiate receptor, the $\text{D}(-)$ isomers should have much higher affinity than the $\text{L}(+)$ isomers. We found that 10^{-7} M and 10^{-6} M levorphanol, a potent opiate that has the $\text{D}(-)$ configuration, decreased the total binding of $[\text{}^3\text{H}]$ naloxone to homogenates of rat, guinea pig, and mouse brain by 70%, while at these concentrations, dextrorphan, the analgesically inactive $\text{L}(+)$ isomer, failed to influence binding. Therefore, in all experiments, incubations with 10^{-7} M concentrations of levorphanol and dextrorphan were included, and the radioactivity in the presence of levorphanol was subtracted as a "blank value" representing nonspecific binding; the failure of dextrorphan to reduce binding in all tissues ensured that the effect of levorphanol was related to its affinity for the specific opiate receptor. In a typical experiment in which 5×10^{-9} M $[\text{}^3\text{H}]$ naloxone (65,000 count/min) was incubated in a 2-mL volume with a homogenate derived from whole rat brain (18 mg, wet weight), the amount bound was 2,000 and 800 count/min in the absence and presence of 10^{-7} M levorphanol, respectively. The term "specific receptor binding" is used to mean the binding of $[\text{}^3\text{H}]$ naloxone in the presence of 10^{-7} M dextrorphan minus its binding in the presence of 10^{-7} M levorphanol.

Specific binding of $[\text{}^3\text{H}]$ naloxone occurred rapidly at 37°C , attained half-maximum values by 2 minutes, and reached equilibrium by 15 minutes. The ratio of specific to nonspecific binding at this time in whole rat brain homogenates was between 2.5 and 3.0 to 1. At 1×10^{-8} M specific binding was half-maximum. With 15-minute incubations specific binding was temperature dependent with maximum binding occurring at 35°C ; a Q_{10} (change of rate of reaction for each 10°C of temperature) value of 1.5 between 25° and 35°C , and with about 90% of the specific binding eliminated at 4°C . Specific bind-

ing was totally prevented by heating homogenates at 55°C for 10 minutes. We found a sharp pH optimum at 7.4 for specific binding with no binding below pH 5 and above pH 10. At 1 mM, calcium and magnesium inhibited stereospecific binding by 50%; up to 500 mM sodium had no influence on binding. Specific binding was not significantly reduced when homogenates were maintained at -20°C for 3 weeks (1).

Dissociation of [³H]naloxone from its binding site was examined at various temperatures by adding 10⁻⁵ M nonradioactive naloxone after a 15-minute incubation with [³H]naloxone. Approximate half-lives for dissociation at 5°, 15°, and 25°C, respectively, were 5 minutes, 2 minutes, and 1 minute (1).

The affinity of drugs for the [³H]naloxone binding sites was examined by incubating tissues with five concentrations of various nonradioactive drugs prior to the addition of [³H]naloxone. The concentration of drug that decreased the specific binding of 5×10⁻⁹ M [³H]naloxone by 50% was determined by log-probit analysis. The slopes of lines describing the percentage of inhibition plotted against drug concentration were the same for all opiates in the brain and guinea pig intestine, respectively (1). The binding affinities of various opiates and antagonists closely paralleled their pharmacologic potencies. Naloxone had an effective dose (ED₅₀) of 1×10⁻⁸ M. Morphine and nalorphine, its corresponding antagonist, showed similar affinities as did levorphanol and its antagonist levallorphan, although in both cases the opiate antagonist was somewhat more potent than the opiate itself. Levorphanol had about 4,000 times the affinity of its nonanalgesic enantiomer dextrorphan. Interestingly, the (-) isomer of methadone, which had 1/10 the affinity of levorphanol, was only ten times more potent than its less analgesically active (+) isomer, conceivably because the methadone molecule has greater conformational mobility than levorphanol (2). Moreover, (-)-levallorphan, whose binding affinity is 20 times that of (-)-methadone, was 5,000 times as potent as its enantiomer in specific receptor binding. Propoxyphene, a weak analgesic (3), had only 1/200 the affinity of morphine for receptor binding. Codeine, which in animals and humans is about 1/10 as active as morphine, bound only weakly to the opiate receptor, being only 1/3,000 as potent as morphine. Since codeine is the methyl ether of morphine and since liver microsomal enzymes are capable of *O*-demethylation of codeine, codeine may exert its analgesic activity only after conversion in the body to morphine (4). Interestingly, while codeine is 1/10 as potent as morphine when administered to the intact animals, it is only 1/100 as potent as morphine in the isolated guinea pig ileum, where the probability of its enzymatic conversion to morphine is greatly diminished (5).

Drugs that are neither opiates nor their antagonists failed to influence [³H]naloxone binding to whole brain homogenates at concentrations of 10⁻⁴ M.

TABLE 1-1. Relative potencies of drugs in reducing stereospecific [^3H]naloxone binding to rat brain homogenates and minced guinea pig intestine

| Drug | ED ₅₀ |
|--|----------------------|
| Brain homogenate | |
| (-)-Naloxone | 1×10^{-8} M |
| (-)-3-Hydroxy- <i>N</i> -allylmorphinan (levallorphan) | 1×10^{-9} M |
| Levorphanol | 2×10^{-9} M |
| (-)-Nalorphine | 2×10^{-9} M |
| (-)-Morphine | 6×10^{-9} M |
| (-)-Methadone | 2×10^{-8} M |
| (\pm)-Pentazocine | 5×10^{-8} M |
| (+)-Methadone | 2×10^{-7} M |
| (\pm)-Propoxyphene | 1×10^{-6} M |
| (+)-3-Hydroxy- <i>N</i> -allylmorphinan | 5×10^{-6} M |
| Dextrorphan | 8×10^{-6} M |
| (-)-Codeine | 2×10^{-5} M |
| Phenobarbital | * |
| Serotonin | * |
| Norepinephrine | * |
| Carbamylcholine | * |
| Choline | * |
| Atropine | * |
| Histamine | * |
| Colchicine | * |
| Intestine, minced preparation | |
| Levorphanol | 8×10^{-8} M |
| (-)-Morphine | 3×10^{-6} M |
| Dextrorphan | 4×10^{-5} M |
| Codeine | 1×10^{-4} M |

*No effect at 10^{-4} M.

Note. Stereospecific [^3H]naloxone binding to rat brain homogenates was assayed as described. Longitudinal muscle with adherent myenteric (Auerbach's) plexus from the small intestine of guinea pigs was minced and suspended in Krebs-Ringer-Tris solution, pH 7.4, at 37°C. Portions (0.4 mL) were transferred to 20-mL beakers containing Krebs-Ringer-Tris solution and the drug (2 mL total volume) and incubated in the same way as brain homogenates. After being cooled, the tissue was filtered over Whatman 540 paper circles and washed with two 8-mL portions of the cold Krebs-Ringer-Tris solution. The filters were transferred to counting vials and shaken for 1 hour at 40°C with NCS (Nuclear-Chicago solubilizer; Amersham/Searle); the radioactivity was determined by liquid scintillation spectrometry after the addition of 15 mL of

TABLE 1-1. Relative potencies of drugs in reducing stereospecific [³H]naloxone binding to rat brain homogenates and minced guinea pig intestine *(continued)*

toluene phosphor. No metabolites of [³H]naloxone in rat brain and guinea pig intestine could be detected after thin-layer chromatographic analysis of ³H-labeled material eluted from tissue by methanol extraction. The ED₅₀ values for inhibition of stereospecific [³H]naloxone binding were defined as the concentration of drug which inhibited by 50% the stereospecific binding of [³H]naloxone (5×10^{-9} M) to whole rat brain homogenate (18 mg, wet weight of tissue) or minced guinea pig intestine (48 mg, wet weight of tissue). Under these conditions binding was linear with tissue protein. The ED₅₀ values were derived from log-probit plots of three to five concentrations of the drug, each assayed in duplicate. Values are the means of three separate determinations.

These include phenobarbital, serotonin, norepinephrine, choline, carbamylcholine, atropine, colchicine, and histamine.

The relative potencies of opiates in reducing [³H]naloxone specific binding in minced guinea pig intestine roughly paralleled results obtained from brain, although the drugs were less potent in the intestine to a variable degree. Levorphanol and morphine were, respectively, 40 and 500 times more active in the brain than in the intestine. In contrast to the 4,000-fold difference in potency between levorphanol and its enantiomer dextrorphan in the brain, these drugs differed only 500-fold in the intestine. This finding suggests that the opiate "receptor" may be qualitatively different in various tissues. Nonetheless, in both brain and intestine, the close parallelism of pharmacological potency and specific binding, as well as the high affinity and stereospecificity of the potent opiates, indicates that [³H]naloxone binds specifically to physiologically significant receptor sites.

Our studies of the tissue distribution of opiate receptor binding provide evidence for the locus of the pharmacologic actions of opiates (Table 1-2). The greatest amount of opiate receptor binding occurred in the brain, while guinea pig small intestine displayed one-half as much opiate receptor binding. However, different binding assays are used for brain and intestine. To ascertain whether the opiate receptor was confined to nervous tissue, we obtained strips of intestinal longitudinal muscle to which the myenteric (Auerbach's) plexus had adhered along portions of its length. Plexus-free portions of the muscle obtained in this way are completely free of innervation (6). When comparable minced samples of plexus-free muscle and innervated muscle were assayed simultaneously, no specific binding at all was detected in the plexus-free muscle. Removal of its innervation in this way abolished all opiate receptor binding from the guinea pig intestine, indicat-

TABLE 1-2. Stereospecific [^3H]naloxone binding in various tissues

| Fraction | [^3H]naloxone specifically bound (dpm/mg) | Percent of total |
|--|--|-----------------------------|
| Rat brain | | |
| Whole brain homogenate | 2,381 \pm 235 | |
| Subcellular | | |
| Crude nuclear | 896 \pm 28 | 21 \pm 3 |
| Mitochondrial-synaptosomal | 2,214 \pm 215 | 48 \pm 1 |
| Microsomal | 3,807 \pm 246 | 32 \pm 2 |
| Soluble supernatant | <250 | |
| Regional distribution | | |
| Striatum | 8,993 \pm 483 | |
| Midbrain | 2,246 \pm 382 | |
| Cortex | 2,089 \pm 395 | |
| Brain stem | 1,282 \pm 63 | |
| Cerebellum | <250 | |
| Guinea pig small intestine | | |
| Myenteric plexus+longitudinal muscle | 1,400 \pm 115 | |
| Longitudinal muscle only (7 mg of protein) | <250 | |
| Human erythrocytes (11 mg of protein) | <250 | |
| Baker's yeast (4 mg of protein) | <250 | |
| Rat liver (6 mg of protein) | <250 | |

Note. For subcellular fractionation, three whole rat brains were homogenized in 20 volumes of 0.32 M sucrose in a glass homogenizer with a loosely fitting Teflon pestle and a portion was removed for receptor-binding assay. The remaining homogenate was centrifuged at 1,000g, this sediment being the crude nuclear fraction. The crude mitochondrial-synaptosomal fraction was derived by centrifuging the supernatant from the crude nuclear fraction at 20,000g for 20 minutes. The microsomal fraction and the soluble supernatant fraction were obtained by centrifuging the supernatant from the mitochondrial-synaptosomal pellet at 100,000g for 90 minutes. The sedimented pellets were suspended in 0.05 M Tris-HCl buffer, pH 7.4 at 37°C, and assayed at five concentrations. After incubation with [^3H]naloxone, protein of the soluble supernatant was precipitated either by ice-cold absolute ethanol, saturated ammonium sulfate solution, or 5% polyethylene glycol; the precipitate was then harvested on a Millipore filter. Values [disintegrations per minute (dpm)] are means of four separate experiments in which specific receptor binding was linear with protein concentration. For ascertaining the regional distribution of binding: Brains from three rats were pooled, and dissected rapidly; the homogenates were assayed at dilutions for which specific receptor binding was linear with protein concentration. The guinea pig intestine was dissected by a modification of the method of Paton and Zar (6).

TABLE 1-2. Stereospecific [³H]naloxone binding in various tissues *(continued)*

Values presented are the means of quadruplicate determinations from two separate experiments in which innervated and denervated muscles were assayed at the same tissue concentrations. Erythrocytes from freshly drawn human blood were assayed both as cell suspensions and as homogenates. The sensitivity of the binding assay is such that 250 dpm of [³H]naloxone per milligram of protein is approximately the minimum amount of binding detectable.

ing that the opiate receptor is confined to nervous tissue. Furthermore, no opiate receptor binding could be detected in human red blood cells and baker's yeast, which are not nerve tissues.

Subcellular fractionation of rat brain showed the greatest enrichment of opiate receptor binding in the microsomal fraction, which is rich in membrane fragments (Table 1-2). About half of the total opiate receptor activity could be recovered in the crude mitochondrial-synaptosomal fraction. The crude nuclear fraction contained the least opiate receptor binding of all fractions examined. No opiate receptor binding could be demonstrated in the soluble supernatant fraction (Table 1-2).

In what type of nervous structures is the opiate receptor localized? Effects of opiates have been demonstrated on acetylcholine (7), serotonin, and norepinephrine (8). Thus, as with most psychoactive drugs, effects can be demonstrated on several neurotransmitter systems so that it is impossible to ascertain which of these represents the primary action of the drug. Our regional study of opiate receptor binding in rat brain revealed the most binding in the corpus striatum, whose binding exceeded that of the cerebral cortex more than fourfold. No opiate receptor binding was detectable in the cerebellum. Of the known neurotransmitters only dopamine and acetylcholine are highly concentrated in the corpus striatum. Dopamine concentrations are low in most other regions of the brain, and its regional variations do not parallel those found for the opiate receptor (9). By contrast, regional differences in acetylcholine concentrations more closely parallel the variations we have observed in opiate receptor binding. Both for opiate receptor binding and acetylcholine concentrations, there is a marked difference between corpus striatum and cerebral cortex, and negligible concentrations of acetylcholine and opiate receptor binding occur in the cerebellum (10). If the opiate receptor is associated with a known neurotransmitter, acetylcholine is the most likely candidate.

Goldstein et al. (11) reported binding of radioactive levorphanol to mouse brain homogenates, about 2% of which showed stereospecific differences between levorphanol and dextrorphan. It is unlikely that the binding

of these two compounds was pharmacologically relevant for the following reasons. Maximum differences between levorphanol and dextrorphan were obtained at 3.9×10^{-3} M, concentrations at which we found that the specific opiate receptor sites are fully saturated for both drugs so that no differences can be detected. Moreover, Goldstein et al. (11) reported that the degree of stereospecificity increased with increasing concentrations of levorphanol and dextrorphan, whereas we found that stereospecific differences decrease with increasing concentrations. Our results differ from those of Goldstein et al. (11) in several other ways. They reported the greatest amount and activity per unit weight of binding in the crude nuclear fraction, whereas we observed the highest specific activity of receptor binding in the microsomal fraction and the greatest recovery in the crude mitochondrial-synaptosomal fraction (Table 1–2). Moreover, they failed to find any regional variations of binding in the brain.

As A. Burgen (12) has pointed out, “Contemporary ideas of drug action and drug specificity are all based on the assumption that the initial process in drug action is the formation of a reversible complex between the drug and a cell component generally known as the drug receptor.” Only drugs that present a high degree of molecular complementarity toward the site at which they act are believed to be able to form this drug-receptor complex (12). We have shown that a component of nervous tissue can selectively form complexes with opiate drugs at very low concentrations. Since this binding is highly stereospecific and corresponds well with the previously reported pharmacological potencies of opiates, we believe that our results represent a direct demonstration of “opiate receptor” binding.

Identification of the opiate receptor provides new insight into the mechanism of action of opiates. Our binding assay affords a rapid means of determining the relative potencies of potential narcotic agonists and antagonists, with attendant theoretical and practical implications.

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CHAPTER 2

Opiate Agonists and Antagonists Discriminated by Receptor Binding in Brain

Candace B. Pert

Gavril Pasternak

Solomon H. Snyder

Opiate antagonists are thought to occupy opiate receptor sites, preventing the access of opiates but not themselves eliciting analgesia or euphoria. Potent, long-lasting, and “pure” antagonists may provide an effective treatment for addiction, while drugs combining opiate agonist and antagonist activities offer promise as non-addicting analgesics (1).

We described opiate binding in animal nervous tissue (2), which we attributed to specific opiate receptor sites on the basis of the stereospecificity of binding (3) and the close parallel between affinity for binding sites and pharmacological potency. These results have been confirmed (4, 5). Of 40 monkey brain regions examined, binding is most enriched in the anterior amygdala, hypothalamus, periaqueductal gray, and caudate head (6). Selective destruction of specific catecholamine, serotonin, and acetylcholine neural pathways in the brain does not alter binding (6). Receptor binding is degraded by trypsin, chymotrypsin, and phospholipase A in concentrations of less than 0.5 $\mu\text{g/mL}$ and by 0.01% concentrations of the detergents Triton-X

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TABLE 2-1. Relative capacities of opiate antagonists and agonists to increase stereospecific [³H]dihydromorphine binding to mouse brain extracts

| Drug | Dose (mg/kg) | Increase (%) |
|------------------------------|--------------|--------------|
| Nalorphine HCl | 0.05 | NS |
| | 0.2 | 37 |
| | 2.0 | 42 |
| | 10.0 | 85 |
| Morphine SO ₄ | 2.0 | NS |
| | 10.0 | 35 |
| | 20.0 | 56 |
| | 50.0 | 67 |
| Naloxone HCl | 0.02 | 11 |
| | 0.2 | 60 |
| | 2.0 | 62 |
| | 20.0 | 74 |
| Oxymorphone HCl | 2.0 | NS |
| | 10.0 | NS |
| | 20.0 | 59 |
| Levallorphan tartrate | 0.1 | 27 |
| | 0.5 | 43 |
| Levorphanol tartrate | 0.5 | NS |
| | 1.0 | 12 |
| | 2.0 | 30 |
| Morphine pellet implantation | | |
| 2 hours | | 43 |
| 12 hours | | 58 |
| 24 hours | | 54 |
| 36 hours | | 40 |
| 60 hours | | 54 |
| 108 hours | | 43 |
| 156 hours | | NS |

Note. Drugs or 0.9% NaCl were injected intraperitoneally, and mice were decapitated 20 minutes later. Before assaying for opiate receptor activity, it was imperative to remove virtually all the nonradioactive morphine, since low concentrations inhibit binding (2). Brain homogenates from control and treated mice were centrifuged at 18,000g for 10 minutes, the supernatant fluid was discarded, and the pellet was re-suspended in 14 mL of cold Tris buffer. This washing procedure was repeated, and after a third centrifugation each homogenate was suspended in 150 mL of the buffer for individual assay. The washing procedure removes virtually all of the ³H in brains of mice treated *in vivo* with [³H]naloxone or [³H]oxymorphone, 1 mg/kg (14 µc). Mor-

TABLE 2-1. Relative capacities of opiate antagonists and agonists to increase stereospecific [³H]dihydromorphine binding to mouse brain extracts *(continued)*

phine pellets (75 mg) were implanted in male Jackson mice (30 g) under light ether anesthesia in the dorsal subcutaneous space (10). Control mice were implanted with placebo pellets composed of the inert ingredients in the morphine pellets. In later experiments, sham-operated mice in which a small incision had been made under anesthesia were used as controls. Values for placebo-implanted, sham-operated, saline-injected, and naive mice are indistinguishable, 24.6 ± 0.4 fmole of stereospecifically bound drug per milligram of protein. All increases are significant ($P < 0.02$) by the Mann-Whitney U rank test. Each value is the mean for one to three experiments. Groups of seven control and seven drug-treated mice were used in each experiment; individual mouse brains were assayed in triplicate with [³H]dihydromorphine (1 nM) in the presence of levorphanol or dextrorphan (100 nM). Values indistinguishable from those shown were obtained when [³H]naloxone was used in the binding assay. NS=not significant.

100, deoxycholate, and sodium dodecyl sulfate, results suggesting that protein and lipid are important constituents of the opiate-receptor complex (7). The receptor is present in all vertebrate brains examined, including mammals, birds, reptiles, amphibia, and teleost fish, but cannot be detected in invertebrates such as arthropods and platyhelminths (8). We now report differential receptor interactions of opiate agonists and antagonists *in vivo* and *in vitro*.

Homogenates of mouse or rat brain (150 mL per gram) were incubated in triplicate in the dark for 30 minutes at 25°C in the presence of 10^{-7} M levorphanol or 10^{-7} M dextrorphan after the addition of the appropriate isotopically labeled opiate or antagonist. Samples were filtered (2) and washed with two 5-mL portions of tris(hydroxymethyl)-aminomethane (Tris) buffer at 4°C. After extraction, the filters were counted by liquid scintillation (2). Specific opiate receptor activity was calculated by subtracting binding of the ³H-labeled ligand in the presence of 10^{-7} M levorphanol from binding in the presence of 10^{-7} M dextrorphan, its analgetically inactive enantiomer, in all experiments except those involving NaCl, in which the antagonist levallorphan and its (+) isomer were substituted for levorphanol and dextrorphan. [³H]Naloxone (23.6 c/mmole), [³H]oxymorphone (0.8 c/mmole), [³H]levorphanol (5.4 c/mmole), [³H]levallorphan (7.6 c/mmole), and [³H]nalorphine (3.4 c/mmole) were obtained from New England Nuclear and purified as described for [³H]naloxone (2). [³H]Dihydromorphine (55 c/mmole) was also purchased from New England Nuclear.

In vivo administration of opiates and their antagonists enhances opiate receptor binding in mouse brain homogenates (Table 2-1). Narcotic antag-

onists are 10 to 1,000 times more potent than their corresponding agonists in enhancing receptor binding. Thus, nalorphine increases receptor binding 37% at a concentration of 0.2 mg per kilogram of body weight, while morphine has no effect at 2 mg/kg and enhances binding by 35% at 10 mg/kg. Naloxone appears to be even more potent compared to oxymorphone, its corresponding agonist. At 0.02 mg/kg, naloxone elicits a statistically significant 11% elevation in receptor binding, with a 60% increase at 0.2 mg/kg. By contrast, oxymorphone produces no effect at 2 mg/kg or 10 mg/kg and a 59% rise at 20 mg/kg. Thus, while nalorphine is 10 to 50 times more potent than morphine, naloxone appears to be 100 to 1,000 times more potent than oxymorphone in enhancing receptor binding. Levallorphan increases binding 27% at 0.1 mg/kg while levorphanol fails to alter binding at 0.5 mg/kg. Enhanced binding is observed as early as 5 minutes after morphine or naloxone treatment and disappears after 2 hours. Measurements of receptor binding at different concentrations indicate that affinity is unchanged but the number of sites appears to increase (9).

To determine whether tolerance and physical dependence are related to alterations in receptor binding, we assayed receptor binding in mice implanted with morphine pellets (Table 2–1), which produce peak tolerance and physical dependence at 3 days (10). Between 2 and 106 hours after pellet implantation, receptor binding is enhanced to a similar extent as in injected mice; binding returns to control levels at 156 hours, when the pellets no longer release morphine (10). Since tolerance and physical dependence in these mice develop gradually, with a maximal fivefold increase in tolerance to morphine at 60 hours, the enhanced receptor binding appears unrelated to tolerance and physical dependence (10).

We evaluated the possibility that enhanced receptor binding is related to generalized behavioral excitation or depression by measuring [^3H]dihydromorphine binding in the brains of mice treated with large doses of reserpine (15 mg/kg daily) for 3 days, sodium pentobarbital (100 mg/kg) for 12 hours, or *d*-amphetamine for 2 days (10 mg/kg on day 1 and 5 mg/kg twice on day 2). None of these treatments alters specific receptor binding.

It has been suggested that opiate antagonists enter the brain more readily than opiate agonists (11). However, these differences are insufficient to account for the large differences in potency described here.

We reported that receptor binding of [^3H]naloxone, an opiate antagonist, was not reduced by sodium (2), while Simon et al. (5) showed that the binding of the agonist [^3H]etorphine was decreased by sodium and potassium; these results suggest possible differential effects of “ionic strength” on agonist and antagonist binding. Incubation with 100 mM NaCl reduces the binding of the opiate agonists [^3H]levorphanol, [^3H]oxymorphone, and [^3H]dihydromorphine by 30% to 70% (Table 2–2). By contrast, binding of

TABLE 2-2. Effects of sodium on binding of ³H-labeled opiate agonists and antagonists to rat brain homogenates

| ³ H-labeled drug | Control stereospecific binding (count/min) | Binding in 100 mM NaCl (% of control) |
|-----------------------------|--|---------------------------------------|
| Agonists | | |
| Dihydromorphine | 2,256±113 | 30 |
| Oxymorphone | 669±32 | 56 |
| Levorphanol | 1,292±61 | 72 |
| Antagonists | | |
| Nalorphine | 408±22 | 145 |
| Naloxone | 1,582±80 | 241 |
| Levallorphan | 2,861±123 | 129 |

Note. Stereospecific binding of tritiated opiates was determined by incubating 1.9-mL portions of washed rat brain homogenate (Table 2–3) in the presence of 100 nM (–)-3-hydroxy-*N*-allylmorphinan (levallorphan) or 100 nM (+)-3-hydroxy-*N*-allylmorphinan. Concentrations of labeled opiates were 1 to 40 nM. Control incubations in the absence of NaCl were performed in 0.05 M Tris-HCl buffer, pH 7.4, at 37°C. Ratios of specific to nonspecific binding ranged from 3:1 to 8:1. Values represent triplicate determinations from a typical experiment that was replicated three times. All tritiated opiates appear to bind to the same population of opiate receptor sites (9).

the antagonists [³H]levallorphan, [³H]naloxone, and [³H]nalorphine is increased 30% to 140%. As little as 0.5 mM NaCl markedly enhances [³H]-naloxone binding, and maximal increase occurs at concentrations around 100 mM. Double reciprocal analysis indicates that sodium increases the number of binding sites with no change in affinity for naloxone. Decrease of [³H]dihydromorphine binding is significant in 5 mM NaCl, while the greatest decrease occurs in 150 mM NaCl, the highest concentration examined. Lithium chloride and the sodium salts Na₂HPO₄ and NaHCO₃ affect binding like NaCl, while potassium, rubidium, and cesium inhibit binding of both agonists and antagonists (9).

It should be possible to use this sodium effect to discriminate opiate receptor binding by nonradioactive agonists and antagonists. In the presence of sodium, opiates of predominantly agonistic nature should have decreased potency in preventing the binding of an isotopically labeled antagonist. By contrast, opiates of predominantly antagonistic nature should have the same capacity to inhibit the binding of antagonists whether sodium is present or absent. We examined the effect of sodium on the capacity of 14 unlabeled opiates to inhibit the specific binding of 1.5 nM [³H]naloxone (Table 2–3).

The pure antagonists naloxone, naltrexone, and diprenorphine displayed no change in affinity for the receptor in the presence of sodium (Table 2–3).

TABLE 2-3. Effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [³H]naloxone binding to rat brain homogenates

| Nonradioactive opiate | ED ₅₀ of stereospecific [³ H]naloxone binding (nM) | | ED ₅₀ ratio, +NaCl/-NaCl |
|-----------------------|--|----------------|--|
| | No NaCl | 100 mM NaCl | |
| Naloxone | 1.5 | 1.5 | 1.0 |
| Naltrexone | 0.5 | 0.5 | 1.0 |
| Diprenorphine | 0.5 | 0.5 | 1.0 |
| Cyclazocine | 0.9 | 1.5 | 1.7 |
| Levallorphan | 1.0 | 2.0 | 2.0 |
| Nalorphine | 1.5 | 4 | 2.7 |
| Pentazocine | 15 | 50 | 3.3 |
| Etorphine | 0.5 | 6 | 12 |
| Meperidine | 3,000 | 50,000 | 17 |
| Levorphanol | 1.0 | 15 | 15 |
| Oxymorphone | 1.0 | 30 | 30 |
| Dihydromorphone | 3 | 140 | 47 |
| Propoxyphene | 200 | 12,000 | 60 |
| Phenazocine | 0.6 | 80 | 133 |

Note. Inhibition of stereospecific [³H]naloxone binding was determined in the presence and absence of 100 mM NaCl for 14 nonradioactive opiates, with (+)- and (–)-3-hydroxy-*N*-allylmorphinan to assess specificity. Rat brain with cerebellum removed was homogenized (Polytron PT-10, 3,000 rev/min) in 100 volumes of 0.05 M Tris buffer and centrifuged at 40,000g for 10 minutes. After the supernatant fluid (which contains no specific binding) was discarded, the pellet was reconstituted in the original volume of Tris buffer. Seven to ten concentrations of each drug were incubated with 1.5 nM [³H]naloxone in the presence and absence of 100 mM NaCl. The concentration of drug that produced a 50% inhibition of control stereospecific binding (ED₅₀) was determined by log-probit analysis. Control [³H]naloxone binding values in the presence and absence of 100 mM NaCl (0.05 M Tris-HCl buffer, pH 7.4, at 37°C) were 1,163±104 and 2,806±198 counts per minute, respectively, at 44% counting efficiency.

By contrast, for the opiate agonists etorphine, levorphanol, meperidine, oxymorphone, dihydromorphone, propoxyphene, and phenazocine, inhibitory potency decreased by a factor of 12 to 133 in the presence of sodium. The three antagonists “contaminated” by agonist properties, cyclazocine, levallorphan, and nalorphine (1), were only slightly affected by sodium, with inhibitory potency decreased by a factor of 1.7 to 2.7 in the presence of

sodium. For pentazocine, considered to be a mixed agonist-antagonist opiate (1), inhibitory potency decreased by a factor of 3.3, a value intermediate between those of predominantly agonist and antagonist opiates.

Under the low sodium condition reported here and previously used (2) in our *in vitro* system, the opiate agonists morphine, oxymorphone, levorphanol, and etorphine have receptor affinities similar to those of their corresponding structurally analogous antagonist derivatives nalorphine, naloxone, levallorphan, and diprenorphine, respectively. Yet opiate antagonists are pharmacologically active at considerably lower doses than agonists *in vivo* (12), a difference not attributable to drug distribution *in vivo*. Indeed, a 10- to 1,000-fold greater potency of antagonists compared to agonists in enhancing receptor binding in mice is reported here.

At the sodium concentrations that normally prevail in the extracellular space of mammalian organisms, antagonists should bind more efficiently than agonists to opiate receptor sites. Thus, the differential influence of sodium on the receptor binding of opiate agonists and antagonists probably accounts for their quantitatively different potencies *in vivo*.

The qualitatively different pharmacological properties of opiate agonists and antagonists may be a reflection of the qualitatively different effect of sodium at receptor sites. The ability to quantify the relative opiate agonist and antagonist activity of drugs by a simple biochemical method may have therapeutic potential for developing "pure" and potent antagonists for treatment of heroin addiction as well as mixed agonist-antagonist drugs that might be non-addicting analgesics.

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CHAPTER 3

Historical Review

Opioid Receptors

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The notion that drugs, hormones, and neurotransmitters act at highly selective recognition sites, designated “receptors,” was reasonably clarified early in the twentieth century and the concept of specific opioid receptors was firmly entrenched more than 35 years ago (1, 2). The extraordinary potency of some opiates, the availability of selective antagonists, and the stereospecificity of opiate actions all favored the existence of specific receptors. Establishing such receptors as specific biochemical entities took longer, particularly for neurotransmitters and drugs that act on them. For the principal biogenic amine neurotransmitters, biochemical features of their synthesis, metabolism, storage in synaptic vesicles, release, and inactivation by enzymes or transporters had been clarified by the early 1970s; however, no one had yet been able to monitor receptors biochemically for any of these neurotransmitters.

A key breakthrough in the biochemical identification of receptors was the development by Lee of α -bungarotoxin (3, 4), an extremely potent, pseudo-irreversible snake toxin that acts selectively at the nicotinic acetylcholine receptor (Table 3–1). Using ^{125}I -labeled α -bungarotoxin, during

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TABLE 3-1. Timeline of opioid receptor research

| Year | Progress made |
|-----------|--|
| 1950s | Clinical studies of nalorphine–morphine interactions |
| 1960s | Proposals and models of opioid receptors |
| 1967 | Receptor dualism |
| 1970–1971 | Demonstration of nicotinic acetylcholine and insulin receptors |
| 1973 | Demonstration of opioid receptors |
| 1973 | Na ⁺ effect: differentiation of agonist and antagonist binding |
| 1975 | Identification of endogenous opioids |
| 1976 | Proposal of mu, kappa, and sigma receptors |
| 1977 | Discrimination of mu and delta receptors |
| 1981 | Demonstration of kappa receptors |
| 1981–1989 | Differentiation of putative subtypes of mu and kappa receptors |
| 1992 | Cloning of the delta opioid peptide (DOP) receptor ^a |
| 1993 | Cloning of the mu opioid peptide (MOP) and kappa opioid peptide (KOP) receptors ^b |
| 1994 | Cloning of the nociceptin/orphanin FQ (NOP) receptor ^c |
| 1990s | Cloning of MOP splice variants |
| 2002 | Cloning of sensory neuron-specific G-protein-coupled receptors |

^aOriginally named DOR-1.
^bOriginally named MOR-1 and KOR-1, respectively.
^cPreviously named ORL1 or KOR-3.

1970 and 1971, three groups identified binding sites that reflected the nicotinic receptor in the electric organ of the electric eel or *Torpedo* (5, 6, 7). Their success was made possible by the extraordinary enrichment of nicotinic receptors in the electric organ of the eel, where the receptors constituted almost 20% by weight of the organ. By contrast, “armchair reasoning” suggested that mammalian brain receptors for most neurotransmitters would probably constitute only a small fraction of a percent of brain protein. In the case of opioid receptors, Vincent Dole, in a classic 1970 review, estimated from relatively rigorous calculations that opioid receptors ought to be only approximately one millionth by weight of the brain (8). The very success of the heroic efforts leading to the identification of the nicotinic receptor seemed to guarantee the impossibility of doing the same for any mammalian brain receptor.

In attempting to understand receptor pharmacology and other fundamental aspects of psychotropic drug activity, opiates are ideal. Over many years, large numbers of opiates had been synthesized with widely varying

potencies and efficacies. The importance of subtle structural differences between agonists and antagonists is evidenced far more extensively with opiates than any other class of drugs. Thus, merely converting an *N*-methyl to an *N*-allyl substituent transforms the agonist morphine to nalorphine, an opioid with antagonistic properties. The same modification of the agonist oxymorphone leads to the pure opioid receptor antagonist naloxone. By contrast, antagonists for most neurotransmitters and other drug classes differ substantially in structure from agonists. Finally, the behavioral correlates of receptor activation, including elements of analgesia, tolerance, and physical dependence and addiction with compulsive drug-seeking behavior, are better characterized with opiates than any other class of drugs.

Identification of Opioid Receptors

In this review, a personalized historical account of early work on opioid receptors and related areas is presented with limited reference to recent developments and suggestions of future challenges (Table 3–1). We focus on an exciting time in opioid research that was shared by several laboratories, in addition to our own, that addressed many key questions in the field in a remarkably short period of time. Thus, in May 1974, little more than a year following the first publications reporting opioid receptor binding, a meeting of the Neurosciences Research Program (9) convened investigators in the field (Figure 3–1). Presentations included detailed descriptions of opioid receptor binding and the first public descriptions of the endogenous opioids.

Our own interest in seeking opioid receptors stemmed from our studies, in collaboration with Pedro Cuatrecasas, on the identification of nerve growth factor receptors (10). This experience provided crucial insights into labeling low-density receptors by reversible ligand binding. The far greater density of nonspecific sites compared with receptor-related binding sites is the biggest hurdle to overcome. First, it is important to use a radiolabeled ligand of high specific radioactivity so that very low concentrations of the radiolabeled ligand can be employed, favoring the labeling of receptors rather than nonspecific sites. Second, preparations must be washed extensively to remove nonspecific binding. Because nonspecific binding sites have poor affinity for the radioligand, the nonspecific labeling will dissociate and wash away far more quickly than will specific binding; thus, rapidly washing the membranes will preserve receptor interactions and lower nonspecific binding. For our washing steps, we used a custom-made filtration manifold with 45 slots for circular filters that was connected to a strong vacuum; this permitted rapid washing with large volumes of buffer.

We sought to apply this approach to opioid receptors for two reasons. First, at that time President Nixon had declared “war on heroin,” which led



FIGURE 3-1. Participants in the Neuroscience Research Program (NRP) Workshop “Opiate Receptor Mechanisms” in Boston, Massachusetts, U.S.A., on 19–21 May 1974.

The workshop was proposed by Frederic Worden, NRP Director, to Solomon Snyder at the November 1973 meeting of the Society for Neuroscience. Efforts were made to include scientists outside the opiate area to facilitate “brainstorming” of the ramifications of opioid receptor research. The proceedings were transcribed and a draft manuscript integrating all presentations and discussions prepared by a group including Huda Akil, Ian Creese, Peter Mansky, and Gavril Pasternak. All participants then provided updating and editing. The final editing was conducted by Solomon Snyder and Steven Matthyse and the volume published in February 1975 (9). Members of the NRP Workshop (left to right): *seated*, Gavril W. Pasternak, William Bunney, John Hughes, Hans Kosterlitz, Steven Matthyse, Francis O. Schmitt, Solomon H. Snyder, Avram Goldstein, E. Leong Way, Vincent P. Dole, and Horace Loh; *middle row*, L. Everett Johnson, Frederic G. Worden, Robert D. Hall, Candace D. Pert, Yvonne M. Homsy, Parvati Dev, Huda Akil, Floyd E. Bloom, Agu Pert, Peter A. Mansky, William H. Sweet, Albert Herz, William R. Martin, and Harriet Schwenk; *top row*, Ian Creese, David J. Mayer, Eric J. Simon, Leslie Iversen, Diana Schneider, Pedro Cuatrecasas, A.E. Takemori, Arnold J. Mandell, Arthur E. Jacobson, Jose M. Musacchio, and Lars Terenius.

to the funding of drug abuse research centers. We received one of the centers with a specific mandate to study opioid receptors (11). Second, we had read a publication by Goldstein et al. (12) that reported the binding of the opiate [^3H]levorphanol to brain membranes with 2% of the binding displaced by the pharmacologically active (–)-isomer but not the inactive (+)-isomer. The radiolabeled levorphanol employed by Goldstein was of low specific activity and extensive washing had not been performed to remove nonspecific binding. When the signal-to-noise ratio in these studies was enhanced, the binding substance appeared to represent the lipid cerebroside sulfate and not the

pharmacologically relevant opioid receptor (13). This seminal paper emphasized stereospecificity as a criterion to identify opioid receptors.

In initial experiments with rat brain we used custom-tritiated naloxone. When combined with the same binding apparatus employed for the nerve growth factor receptor research, these experiments clearly demonstrated saturable binding, with 70% of the total binding being stereospecific for the isomers of levorphanol and manifesting high affinity for established opioids (Table 3–2) (14). The high signal-to-noise ratio and the ability to assay dozens of samples simultaneously enabled us to evaluate drug specificity and other properties rapidly and efficiently. In addition, interesting pharmacological questions could be addressed. For example, codeine, which is 3-O-methylmorphine, was virtually inactive at the binding sites. This fitted with the notion that codeine acts only after being demethylated by cytochrome P450 enzymes in the liver, with the resulting morphine gradually entering the brain, possibly explaining the reduced “high” and lesser abuse potential of codeine compared with morphine. In intestinal preparations, we showed that the receptors were exclusively localized in the myenteric plexus of neurons (14, 15), consistent with evidence from pharmacological studies.

Independently, Terenius had been studying the transport of [^3H]dihydromorphine into nerve terminal preparations (synaptosomes) of guinea-pig ileum (16). Using brain membrane preparations to examine the recognition site, he observed saturable binding that was inhibited stereospecifically by levorphanol isomers, and subsequently showed that the recognition site possessed properties of opioid receptors (17, 18). Employing [^3H]etorphine of high specific radioactivity and the incubation procedures that we had reported (14), Simon and associates also identified saturable opioid receptor binding sites (19).

The ability to label opioid receptors with reversible ligands suggested that, with the appropriate ligand of high potency labeled to high specific radioactivity, one might be able to identify receptors for the major neurotransmitters in the brain. Indeed, in an elegant series of studies about a year following opioid receptor identification, Lefkowitz and colleagues characterized β -adrenoceptors using [^3H]dihydroalprenolol (20). Soon after our initial opioid receptor experiments, we used [^3H]strychnine, known to block the effects of the inhibitory neurotransmitter glycine, to label glycine receptors (21). Furthermore, the very potent anticholinergic drug [^3H]quinuclidinyl benzilate was used to label muscarinic acetylcholine receptors (22), and dopamine receptors were labeled with the neurotransmitter itself [^3H]dopamine in addition to [^3H]haloperidol (23), findings obtained independently by Seeman (24). GABA receptors could be labeled with [^3H]GABA (25, 26) and 5-HT receptors were labeled with [^3H]5-HT, [^3H]lysergic acid diethylamide (LSD), or [^3H]spiperone (27). Thus, within 3–4

TABLE 3-2. Drug competition for [³H]naloxone binding^a

| Drug | ED ₅₀ (nM) |
|--|-----------------------|
| (-)-Naloxone | 0 |
| (-)-3-Hydroxy- <i>N</i> -allylmorphinan (levallorphan) | |
| Levorphanol | |
| (-)-Nalorphine | 2 |
| (-)-Morphine | 6 |
| (-)-Methadone | 20 |
| (±)Pentazocine | 50 |
| (+)Methadone | 200 |
| (±)Propoxyphene | 1,000 |
| (+)3-Hydroxy- <i>N</i> -allylmorphinan | 5,000 |
| Dextrorphan | 8,000 |
| (-)-Codeine | 20,000 |

^aThis initial characterization of drug influences on opioid receptor binding illustrates the power of reversible ligand binding to characterize pharmacological actions, which was a novel approach in the early 1970s. Stereospecificity of the receptor is evident by the 4,000-fold greater potency of levorphanol than its enantiomer dextrorphan. The inactivity of codeine (3-*O*-methylmorphine) indicates that the drug acts only following its *O*-demethylation by hepatic cytochrome P450 enzymes to morphine, providing more gradual access to the brain and hence lesser euphoria. The similar potencies of the agonist morphine and the corresponding antagonist nalorphine (which differs from morphine by transformation of an *N*-methyl to an *N*-allyl substituent) establish the challenge of differentiating agonists and antagonists. Phenobarbital, 5-HT, noradrenaline, carbamylcholine, choline, atropine, histamine, and colchicine have no effect at 100,000 nM.

Source. Adapted, with permission, from Pert and Snyder (14). ©1973 American Association for the Advancement of Science (<http://www.sciencemag.org>).

years of the first opioid receptor labeling, this binding paradigm had been extended to all of the principal neurotransmitter receptors.

Differentiating Agonists and Antagonists: Links to Second Messengers

Because of therapeutic implications, differentiating agonists from antagonists is particularly important with opiates. Although nalorphine is a potent opioid receptor antagonist and was initially employed as an antidote to opiate overdose, Lasagna (28) and Houde (29) independently discovered in clinical studies that it possessed agonist activity. Thus, low doses of nalorphine reversed the actions of morphine but analgesia returned with higher

doses of nalorphine. However, dysphoric effects, probably mediated through kappa opioid receptors, precluded the widespread clinical use of nalorphine. These findings spurred the pharmaceutical industry to develop mixed agonist–antagonists as potentially less addicting opiates, with pentazocine (Talwin) being the first marketed agent of this type.

In our initial experiments, the receptor binding affinities of morphine and nalorphine were indistinguishable, as were several other matched pairs of *N*-methyl (agonist) and *N*-allyl (antagonist) opioids. However, on examination of ionic influences, we noted that Na^+ ions increased [^3H]naloxone binding by 45%, but decreased [^3H]dihydromorphine binding by 70% (30). Simon also had observed inhibitory effects of Na^+ on the binding of the agonist [^3H]etorphine (19). We examined the influence of Na^+ systematically by monitoring the potencies of unlabeled drugs to inhibit [^3H]naloxone binding in the absence or presence of Na^+ and found dramatic variations (30). Na^+ failed to influence the potencies of pure antagonists but decreased the potencies of pure agonists by up to 99% (Table 3–3). Mixed agonist–antagonists, which were less addicting analgesics, were affected in an intermediate fashion. Thus, within a year of the first labeling of opioid receptors, drugs could be differentiated on an agonist–antagonist continuum.

One implication of these findings involved drug development. Chemical modifications that differentiated mixed agonist–antagonists from pure antagonists had been mystifying to researchers and there was no way of predicting function from structure except for performing experiments in intact rodents. Screening with intact mice or rats permitted evaluation of only a handful of drugs a week and required chemical synthesis of gram quantities. By contrast, ligand binding studies, in which a technician in 1973 could examine 1,000 test tubes in a day, permitted screening of much greater numbers of substances, required only microgram amounts of candidate drugs, and could predict the agonist and/or antagonist character of the agent. Those principles of receptor binding are still the standard approach to drug development in the pharmaceutical industry, with robotic modifications routinely screening 100,000 or more chemicals at individual receptor targets.

From the standpoint of receptor pharmacology, molecular discrimination of agonists and antagonists might clarify how recognition of a drug or neurotransmitter at its receptor impacts intracellular events. At the time of our initial characterization of the “sodium effect” G proteins were not yet appreciated. Subsequently, when it became evident that GTP binding to membrane preparations involved G proteins with GTP selectively decreasing the affinity of agonists, we found that Na^+ and GTP affected opioid receptor binding synergistically (31).

Na^+ was subsequently shown to differentiate agonists and antagonists at numerous G-protein-coupled receptors. Focusing on the α_2 -adrenoceptor,

TABLE 3-3. Differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions^a

| Nonradioactive opiate | IC ₅₀ (nM) | | IC ₅₀ ratio |
|-----------------------|-----------------------|-------------|------------------------|
| | No NaCl | 100 mM NaCl | |
| Naloxone | 1.5 | 1.5 | 1.0 |
| Naltrexone | 0.5 | 0.5 | 1.0 |
| Diprenorphine | 0.5 | 0.5 | 1.0 |
| Cyclazocine | 0.9 | 1.5 | 1.7 |
| Levallorphan | 1.0 | 2.0 | 2.0 |
| Nalorphine | 1.5 | 4.0 | 2.7 |
| Pentazocine | 15 | 50 | 3.3 |
| Etorphine | 0.5 | 6.0 | 12 |
| Meperidine | 3,000 | 50,000 | 17 |
| Levorphanol | 1.0 | 15 | 15 |
| Oxymorphone | 1.0 | 30 | 30 |
| Dihydromorphone | 3.0 | 140 | 47 |
| Propoxyphene | 200 | 12,000 | 60 |
| Phenazocine | 0.6 | 80 | 133 |

^aThe discrimination of receptor binding for opioid agonists and antagonists was initiated by the observation that binding of the agonist [³H]dihydromorphone was decreased by Na⁺ ions whereas the binding of the pure antagonist [³H]naloxone was increased by 40%. We suspect that [³H]naloxone binding increased because Na⁺

Limbird and colleagues demonstrated that Na⁺ acts on a single amino acid in the receptor, Asp79, to influence ligand binding and G-protein–effector coupling (32). However, it still is not clear how Na⁺ or other monovalent cations in microdomains associated with receptors physiologically regulate receptor conformation.

The influence of Na⁺ on opioid receptor binding established that links between receptors and second messengers can be preserved in crude membrane preparations. This notion was supported in our studies of glycine receptors labeled with [³H]strychnine (33). Glycine hyperpolarizes neurons by opening Cl[−] ion channels, and the relative ability of anions to penetrate the channel is well characterized. Cl[−] inhibited [³H]strychnine binding, and the relative potencies of anions in influencing binding paralleled closely their ability to penetrate the glycine-linked Cl[−] channel. Similarly, the binding of Ca²⁺ antagonist drugs to their receptors was influenced by Ca²⁺ and other divalent cations in proportion to their ability to penetrate the relevant Ca²⁺ channel (34). In addition to influencing opioid receptor binding, GTP modulates ligand binding to virtually all G-protein-coupled receptors.

TABLE 3-3. Differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions^a (*continued*)

caused endogenous opioid peptides to dissociate from the receptor making more receptors accessible to [³H]naloxone (37, 45). Custom tritiating an extensive series of opioid agonists and antagonists to evaluate multiple drugs would be prohibitively expensive. A simple solution was to monitor [³H]naloxone binding comparing potencies of drugs in the presence or absence of Na⁺. Binding of the “pure” antagonists naloxone, naltrexone, and diprenorphine was unaffected by Na⁺, whereas “pure” agonists became up to 99% less potent in the presence of Na⁺. Mixed agonist–antagonists were affected in an intermediate fashion. At the time of these studies the existence of multiple receptors was not known. It is likely that some of the variability in the influences of Na⁺ on potency reflected differential sensitivity of receptor subtypes to Na⁺. G proteins had also not yet been identified so that the mechanism of action of Na⁺ was a mystery, although it clearly was influencing links between ligand binding at the plasma membrane and intracellular second messenger systems. We now know that the “Na⁺ effect” reflects the influences of Na⁺ on multiple G-protein-coupled receptors (32). The Na⁺ effect on receptor binding provided a simple and sensitive means for discriminating a continuum of agonists and antagonists and came to be employed extensively by the pharmaceutical industry for drug development. Drug potencies for inhibiting [³H]naloxone binding to rat brain membranes were assessed in the presence or absence of 100 mM Na⁺. IC₅₀ values were calculated as the concentration required to reduce [³H]naloxone binding by 50%.

Source. Adapted, with permission, from Pert et al. (30). ©1973 American Association for the Advancement of Science (<http://www.sciencemag.org>).

Agonist and antagonist binding to opioid receptors can also be discriminated in several other ways. In contrast to Na⁺ ions, many divalent cations, such as magnesium and manganese, enhanced opioid agonist binding, with chelators showing an opposite effect (35). Mild digestion with sulfhydryl reagents (36, 37) or proteolytic enzymes selectively decreased agonist binding (38). Even the temperature of the binding assay influenced the binding of agonists and antagonists differently, with lower temperatures being more conducive for antagonist binding (39). Together, these studies revealed marked differences in the stability of agonist and antagonist conformations of the receptor.

Receptor Localization

In our very first experiments, we found major regional variations in [³H]naloxone binding, with negligible binding in the cerebellum and highest levels in the corpus striatum (caudate–putamen). The levels in the caudate–putamen were more than four times the levels found in the cerebral

cortex and midbrain, and seven times the levels found in the brain stem (14). The very high levels of opioid receptors that were observed in the caudate–putamen enabled, 12 years later, the imaging of receptors in human subjects by positron emission tomography (PET) scanning (40). In a detailed dissection of monkey brain, in collaboration with Kuhar, we uncovered even more dramatic variations that could explain many of the pharmacological actions of opiates (41, 42). Receptors were greatly enriched in the periaqueductal gray, where electrical stimulation produces analgesia that is blocked by naloxone (43, 44). The medial thalamus, which conveys to the cerebral cortex information about “emotional” components of pain, had almost four times the density of receptors than the lateral thalamus, which handles touch sensation and the “pin prick” type of pain that is not influenced by opiates. Limbic regions of the brain, such as the amygdala, which regulate emotional behavior, also possessed a high opioid receptor density. Similar variations in opioid receptor binding were observed by Simon’s group (45).

The most important advance in localizing receptors came with the development by Kuhar and associates of autoradiographic techniques for localizing receptors after first administering the radiolabeled ligand to the intact animal (46) and subsequently, and more importantly, following incubation of the radioligand with brain slices (47). Autoradiography revealed extremely dense concentrations of opioid receptors in nuclei such as the locus coeruleus, the source of the major noradrenaline-containing cell bodies in the brain; the substantia gelatinosa of the spinal cord and brain stem, the first site for integrating sensory information within the CNS; and vagal nuclei such as the nucleus ambiguus and nucleus solitarius. These “hot spots” for opioid receptors correlated very closely with the localization of enkephalin-containing neurons (48, 49), and provided some of the first robust evidence that the enkephalins are endogenous ligands for the opioid receptor.

Multiple Receptors

The concept of multiple opioid receptors long preceded their identification as biochemical entities. In clinical studies of nalorphine pharmacology in humans, Lasagna and Beecher (28) and Houde and Wallenstein (29) noted that low nalorphine doses could block morphine analgesia, but that at higher doses of nalorphine analgesia returned, findings that could only be explained by influences on a second receptor. In the late 1960s Martin (50) used these studies, which had subsequently been replicated in animal models, to propose distinct receptors for morphine (M receptor) and nalorphine (N receptor), a concept he labeled “receptor dualism.” More detailed studies using a series of benzomorphan led him to further refine the concept of

multiple opioid receptors and suggest the existence of distinct receptors, designated kappa, which mediate the actions of ketocyclazocine, and mu, which are activated by morphine (51). Martin also suggested that the psychotomimetic actions of certain opiates involved a receptor he designated "sigma" (51). We now know that the psychotomimetic effects of phencyclidine reflect blockade of the NMDA subtype of glutamate receptors. Sigma binding proteins, subsequently purified and cloned (52, 53, 54), do not appear to represent pharmacologically defined opioid receptors.

Although pharmacological studies in intact animals were consistent with multiple receptors, one could equally well conclude that differences between drugs simply reflected actions by certain drugs on sites unrelated to opioid receptors. Definitive demonstration of the three major families of receptors came from ligand binding studies in addition to investigations in isolated nerve-smooth-muscle preparations, although the subsequent synthesis of selective mu opioid peptide (MOP), delta opioid peptide (DOP) and kappa opioid peptide (KOP) receptor antagonists has proven valuable (55, 56, 57). Soon after the identification of the enkephalins, binding studies were undertaken with the tritium-labeled peptides. We found differences in the relative potencies of drugs in competing for [3 H]enkephalin and [3 H]naloxone binding (58, 59). We erroneously interpreted the discrepancies as related to the possibility that the enkephalins, five-amino-acid peptides, would bind at more sites on the receptor protein than [3 H]naloxone, leading to varying drug potencies in displacing individual amino acids of enkephalin bound to the receptor. Kosterlitz and colleagues obtained results similar to our own, but in addition conducted studies in the guinea-pig ileum and mouse vas deferens to examine the ability of drugs to influence electrically induced contractions of these preparations (60, 61, 62). They found that the relative potencies of drugs in the vas deferens paralleled their potencies in competing for [3 H]enkephalin binding whereas contractile potencies in the ileum correlated with potencies in competing for [3 H]naloxone binding (61, 62). Receptors associated with the vas deferens were designated DOP receptors, whereas those that favored the ileum preparation corresponded to the morphine-selective MOP receptor sites.

To find the postulated KOP receptors, the Kosterlitz team examined [3 H]ethylketocyclazocine {[3 H]EKC} binding in the guinea-pig cerebellum and identified binding sites with the pharmacological properties of KOP receptors (63). Lacking a simple smooth muscle system to evaluate pharmacological activity, they alkylated brain membranes with phenoxybenzamine and examined the ability of unlabeled MOP-, DOP- and KOP-receptor-specific drugs to protect putative receptor subtypes from degradation. These experiments gave evidence for biochemically distinct MOP, DOP and KOP receptors. More definitive demonstration of KOP receptor binding sites

came with the synthesis of highly selective KOP receptor ligands, such as U50488H (64).

Although the binding studies identifying the three principal receptor subtypes were relatively crude, subsequent cloning has revealed that Kosterlitz had successfully labeled the three major subtypes of opioid receptors. One of the main reasons for seeking receptor subtypes was the hope that one of them might mediate analgesic actions and another might mediate the addictive properties of opiates. In recent years, mice with the genetic knockout of MOP, DOP and KOP receptors have been generated (65, 66, 67, 68, 69). Although all permutations and combinations of cross-bred receptor knockouts have yet to be characterized in detail, some general patterns have emerged (70). Knockout of MOP receptors [MOR-1 (the first MOP receptor to be cloned)] markedly diminishes virtually all morphine actions but does not affect spinal DOP- or KOP-receptor-mediated analgesia, although supraspinal DOP- or KOP-receptor-mediated analgesia is modestly diminished (71, 72).

Opioid Peptides

The properties of opioid receptors resembled those of receptors for neurotransmitters to such an extent that one immediately wondered whether endogenous ligands for these opioid receptors might exist. The existence of such endogenous ligands was already suggested following studies of brain-stimulated analgesia. For example, Liebeskind and colleagues had shown that brain stimulation in certain areas, particularly the periaqueductal gray, caused pronounced analgesia (43), which was blocked by naloxone (73).

Initial attempts to identify endogenous opioids took two routes. In our own laboratory, Pasternak found that simply incubating brain tissue resulted in significantly enhanced receptor binding as a result of the release of an endogenous heat-stable inhibitor of opioid receptor binding into the supernatant (37). Additionally, protein-free brain extracts inhibited opioid receptor binding (9, 74, 75). Specificity was ensured by the observation that the relative densities of this opioid-like material in various brain regions closely paralleled variations in the densities of opioid receptors. Terenius also found that brain extracts could inhibit [^3H]opioid binding to receptors (9, 76). Hughes and Kosterlitz noted that brain extracts mimicked the ability of morphine to inhibit electrically induced contractions of the mouse vas deferens, with these effects being blocked by naloxone (9, 77). In December 1975 they published the isolation and amino acid sequence of the two enkephalin pentapeptides, methionine-enkephalin {Tyr-Gly Gly Phe-Met; [Met(5)]enkephalin} and leucine-enkephalin {Tyr-Gly Gly Phe-Leu; [Leu(5)]enkephalin} (78), whereas we obtained the same sequences a few months later (79).

Before the isolation of the enkephalins, in the summer of 1975 some participants at an opiate meeting at Airlie House in Virginia caucused to name the hypothetic endogenous opioids. The consensus selection, put forward by Eric Simon, was “endorphine” for “endogenous morphine.” Subsequently Avram Goldstein, in round-robin correspondence, argued that endogenous ligands such as serotonin and heparin lacked an “e,” so he recommended changing the designation to endorphin (11). Because Hughes and Kosterlitz were the first to isolate and sequence the first opioid peptide, they had the privilege of selecting a name. They did not wish to focus exclusively on opiate-like properties, and so selected “enkephalin” from the Greek meaning “in the head” instead of the term “morphine-like factor,” which was suggested independently by Terenius and Pasternak. Ironically, the highest enkephalin levels in the body were subsequently found within the adrenal gland.

The sequence of [Met(5)]enkephalin is also contained in the sequence of the pituitary protein β -lipotropin, identified by C.H. Li (80). A 31-amino-acid portion of this protein, designated β -endorphin (81), possesses high affinity for opioid receptors. Subsequently, a series of peptides incorporating the leucine–enkephalin sequence were isolated by Goldstein and associates (82) and designated the dynorphins. Cloning of the peptide precursors revealed one precursor containing both [Met(5)]enkephalin and [Leu(5)]enkephalin [83], a second precursor for the dynorphin peptides (for review, see [84]) and yet a third precursor for β -endorphin and the other pro-opiomelanocortin (POMC) peptides adrenocorticotrophin and melanocyte-stimulating hormone (84). These cloning studies also identified several other potential opioid peptides containing the sequences of either [Met(5)]enkephalin or [Leu(5)]enkephalin.

Future Challenges

Non-addicting Opiates

Understanding addiction–tolerance definitively at a molecular level has resisted the efforts of many investigators. As soon as opioid receptor binding was identified we looked long, hard and unsuccessfully for relevant changes in receptors and made similarly futile efforts to link enkephalin levels to addiction (85). When the enkephalins were first identified, pharmaceutical companies reasoned, “we aren’t addicted to ourselves,” and so enkephalin derivatives might afford non-addicting analgesics. Yet, early studies showed that enkephalins could produce tolerance (86). Some investigators still believe that delta opioids might provide analgesics with less addictive potential than do heroin and other mu opioids. Much effort has been devoted to KOP receptor agonists because stimulation of KOP receptors seems to provide

less tolerance and physical dependence than activation of other receptor subtypes (87). However, in clinical trials most KOP receptor agonists have been dysphoric and psychotomimetic (88), suggesting that the “sigma activity” initially proposed to elicit psychosis might reflect KOP receptor effects. Mixed agonist–antagonists have consistently been somewhat less addicting, perhaps because they typically retain KOP receptor activity.

Conversely, drugs that counter the addictive actions of opioids would be desirable. Although mechanisms that regulate self-administration and addiction remain unresolved, there is evidence that it might be possible to develop antagonists that are capable of selectively blocking reward mechanisms. A derivative of naltrexone, 3-methylnaltrexone, is an opioid receptor antagonist that blocks the analgesic and rewarding actions of heroin at doses that do not interfere with morphine-induced analgesia (89). Although its selectivity is modest, it does raise the possibility of antagonists that are selective for addiction.

Functions of Opioid Peptides

Considering the different potential opioid peptides that are anticipated from the cloned precursors of the enkephalins, dynorphin and β -endorphin, dozens of opioid peptides now exist (84). Are discrete functions served by each of so many distinct opioid peptides? These are not simple issues to address. Indeed, it is not even certain that these extended enkephalins and dynorphins are functionally significant. However, the recent discovery of the sensory neuron-specific G-protein-coupled receptors that bind proenkephalin A with high affinity (90) raises the possibility of even more receptors for the endogenous opioids.

Concluding Remarks

Science progresses in fits and starts with frequent reassessments and corrections of earlier errors. The opioid receptor field is notable in how the earliest crude biochemical findings have held up under the scrutiny of modern molecular biology. The opioid binding sites identified in 1973 remain the mediators of the major pharmacological actions of opiates. The subtypes of MOP receptors suggested by pharmacology in intact animals and by limited binding studies mesh remarkably well with receptor subtypes that have been cloned subsequently. The initially identified opioid peptides remain accepted as neurotransmitter ligands for opioid receptors. Nonetheless, some of the most crucial challenges, understanding mechanisms of addiction and the development of non-addicting opiates, remain unfulfilled.

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Part II

CHARACTERIZATION OF THE **E**NKEPHALINS

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COMMENTARY

The Discovery of Endogenous Opiates and Their Receptors: A Snyderian Saga of Skill and Judgment

Charles B. Nemeroff

My e-mail response to Sol Snyder's invitation to write this commentary on his seminal contributions to the discovery of endogenous opiates and their receptors required no more than a few nanoseconds. It provided me with the delightful opportunity to reminisce about Sol Snyder, the person and the scientist—I hope conveying to the reader what a remarkable and unique role he has played in the still-burgeoning field of neuroscience, with an emphasis on the pathophysiology and treatment of major psychiatric disorders. Equally, he has played a unique role as mentor to a legion of students. This brief essay focuses on two major themes: Sol's personal attributes and their enormous contribution to his success, and the importance of his work on the endogenous opioid system.

I first met Sol Snyder 34 years ago when I was a first-year graduate student at the University of North Carolina in Chapel Hill and he was a visiting professor in our neurobiology program. In addition to giving the requisite lecture, visiting professors met with neurobiology graduate students without any faculty present, and our meeting with Sol was held at a local tavern, the Rathskeller. Over more than one pitcher of beer, Sol entertained and educated us, and even now, so many years later, I can recall my first impressions of him. He was a remarkably attentive listener (his psychiatric training?) to the students, no matter how seemingly mundane our questions were, and I finally realized that one of Sol's great strengths was listening to students, as

well as technicians, janitors, taxi drivers, and anyone who shared his interest for novel ideas. He intuitively grasped the notion that students, particularly early in their careers, were not dogmatized by conventional (i.e., often incorrect) wisdom. He is also the quintessential anti-snob. In his interactions with this group of first- and second-year graduate students, he dropped no names of the rich and famous he routinely associated with, and most remarkably learned all of our names in short order. He was also not defensive about his own work and took in stride his involvement in scientific controversies. He emphasized that sometimes even he was not always correct in his conclusions. He reminded us that far worse than having work challenged is having it ignored. Like his mentor, Julius Axelrod, he encouraged us to “take a flyer,” that is, take chances, occasionally engage in high-risk research. More recently, he has summarized these ideas in his essay on “audacity” (1; Chapter 27 in this volume). That night, Sol was in perpetual motion and seemed to my inexperienced eye to be a great scientist and a great guy, a bit of a “science nerd.” Little did I know at the time of his talents on the tennis court, his accomplishments with the classical guitar, and his devotion to the Baltimore Orioles.

In the thirty-plus years that followed, I have had the opportunity to visit with Sol on a number of occasions, to review his grants and papers, to contribute to volumes he has edited, to read his popular books, and to meet some of his family. What emerges is a man first and foremost loyal to friends and family, a true frontiersman, a *mensch* of the highest order, a phenomenally productive scientist who has achieved, by orders of magnitude, more than most yet somehow has developed a correct balance between family, outside interests, and work. It is important to note his capacity for opening up entire new fields—he is the consummate trailblazer, the antithesis of the slow incremental plodder. Many in our field, myself included, have tried to adopt this very style. Sol’s sense of humor and ability to laugh at himself are legendary—I remember sitting next to him at a scientific advisory board meeting, and I could tell that he was bored and having difficulty sitting still. He tapped me on the shoulder, and when I faced him, he opened his hand containing 25 or more different pills and capsules of different shapes, colors, and sizes, and asked if I wanted any! At another meeting of the same group, Sol, again fidgeting, stuck a pencil in his ear and the eraser ended up being lodged in his ear canal—necessitating an emergency room visit to effect its retrieval.

Prior to a discussion of the three original research articles on opioids, I wish to point out a glaring and egregious oversight—the lack of a Nobel Prize on his curriculum vitae. Considering his manifold contributions and phenomenal number of published reports, particularly in *Science* and *Nature*, this omission is not understandable. If considerations other than purely

scientific ones have contributed to his being overlooked in this regard, it represents the cruelest form of irony, because Sol is one of the most egalitarian physician-scientists I have ever met, simultaneously upholding the highest scientific standards and having not one shred of prejudice toward any group. It is, of course, not too late for this sorry situation to be rectified.

Finally, of the work itself. I again return to that first meeting 33 years ago, before his (and others') discovery of endogenous opioids or their receptors. Sol leaned across that old wooden table in the Rathskeller and asked me if I thought that God had invented the poppy so that man could become either pain free or addicted to opiates. Alternatively, did I think that endogenous opioids existed? I remember babbling about a recent article I had read in a very obscure journal on the presence of benzodiazepine-like substances in postmortem human tissue collected prior to the availability of diazepam or chlordiazepoxide. The three reports reprinted here were published in three consecutive years (1975, 1976, 1977). The first (Chapter 4) was a review article published in *Nature* based on a lecture delivered at the Massachusetts Institute of Technology (2). It summarized the work of Sol's group and others identifying and characterizing opiate receptors in the central nervous system and his belief that endogenous opioids, not yet discovered, acted on these receptors. Moreover, he posited a critical role for this system in pain perception and its treatment, as well as in drug addiction. The second paper (Chapter 5), published in *PNAS* in 1976, confirmed the existence of two endogenous opioid peptides, met- and leu-enkephalin (3), first reported by Hughes and colleagues. The final report (Chapter 6), published in *PNAS* in 1977, used immunohistochemical techniques to demonstrate the heterogeneous distribution of these two enkephalins in the rat central nervous system (4). More specifically, it documented the presence of enkephalin-containing nerve terminals in brain regions known to modulate pain—such as the spinal cord and periaqueductal gray, the brain areas where enkephalins were found in close proximity to opiate receptors—as well as areas in which so-called mismatch occurred. It is difficult to overemphasize the importance of these reports and their impact on the field—and perhaps hard to quite grasp it today in the context of our considerable understanding of the importance of neuropeptide neurotransmitters in CNS function. However, these reports were published in an era when classical neurobiologists argued vociferously that neuropeptides did not meet the criteria for neurotransmitter status (5), and this view was often supported by the then-emerging new discipline of neuroendocrinology, which viewed neuropeptides such as the hypothalamic releasing factors as functioning largely, if not solely, as neurohormones.

I am grateful to Sol for the opportunity to provide this commentary and, more importantly, to thank him on behalf of our entire field for all of his contributions.

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CHAPTER 4

Opiate Receptor in Normal and Drug Altered Brain Function

Solomon H. Snyder

Extracts of the poppy plant have been used since the days of the Homeric epics medically and recreationally to relieve pain, induce sleep, ease anxiety, for diarrhea, heart failure, and simply to promote a sense of well being. It has long been known that opiates can be addictive and a major goal of opiate research is to develop a nonaddictive opiate analgesic. Although non-opiate pain killers such as aspirin are useful in some situations, only opiates seem to be effective in the treatment of severe and protracted pain. Besides such a practical goal, understanding how opiates act may elucidate fundamental questions in pharmacology. Opiates provide the classic paradigm for tolerance and physical dependence, which can occur to a variety of drugs such as alcohol and barbiturates.

What opiates might tell us about normal brain function is a question that prompts consideration of the opiate receptor itself. The fairly stringent chemical requirements for opiate activity, the fact that opiate effects are highly stereospecific and that some opiates, such as etorphine, are so potent that they act in doses even lower than LSD, all suggest that opiate actions must involve highly selective sites or “receptors” in the brain. The existence

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This paper derives from the second F. O. Schmitt Lecture in Neurosciences delivered by the author at the Massachusetts Institute of Technology, October 1974. Studies described here were conducted in collaboration with C.B. Pert, G.W. Pasternak, I. Creese, and M.J. Kuhar.

of opiate antagonists also favors the receptor concept. Opiate antagonists are drugs, closely related in structure to the corresponding opiate analgesics, which may have little or no analgesic or euphoric effect themselves but which can completely reverse action of opiate analgesics. It seems as if the antagonists occupy opiate receptor sites, doing nothing themselves, but preventing access of opiate analgesics. If specific opiate receptors exist in the brain, one would assume that they were created for some normally occurring substance. Indeed, researchers have identified a normally occurring morphine-like factor in the brain with high affinity for specific opiate receptor sites. The distributions of the morphine-like factor and the opiate receptor itself correspond in part to certain pain pathways in the brain, suggesting that the morphine-like factor may be a neurotransmitter for pathways in the brain mediating pain and emotions. A detailed understanding of such systems might help elucidate some riddles of addiction.

Demonstrating the Opiate Receptor

The most direct way to identify receptor sites is to measure the binding of radioactive drugs to brain tissue. Like most chemicals, however, opiates bind to almost any biological or nonbiological membrane so that nonspecific binding, not associated with the receptor, greatly exceeded any receptor binding in most early studies. Goldstein et al. (1) enunciated the criterion that pharmacologically relevant opiate receptor binding should, like opiate analgesic effects themselves, be stereospecific.

In our laboratory, we developed simple technical maneuvers to amplify specific receptor binding and decrease nonspecific binding. Thus, if opiates bind with considerable affinity to their receptor sites, the use of small amounts of drug labeled to high specific radioactivity favors specific, as opposed to nonspecific, binding, whereas washing tissue thoroughly but rapidly after binding preferentially removes nonspecific binding. On this basis, specific opiate receptor binding has been demonstrated to brain and guinea pig intestine (2, 3, 4, 5).

But stereospecific binding itself is not a sufficient criterion for the pharmacologically relevant opiate receptor. Some glass filters (6) and numerous acidic lipids which may or may not be related to the biological receptor (7, 8) bind opiates stereospecifically. To ensure that binding to brain membranes represents receptor interactions that operate *in vivo*, many drugs were shown to exhibit affinity for the opiate receptor which closely parallels analgesic potency, while non-opiate drugs have negligible affinity for the opiate receptor. Ideally, one should compare pharmacological potency and receptor binding in the same system, which is feasible because the ability of opiates to inhibit electrically induced contractions of the guinea pig intestine closely

parallels analgesic activity (9, 10). Affinity of both opiate agonists and antagonists for receptor binding sites is closely similar in brain and guinea pig intestine and pharmacological and binding potencies of many opiate agonists and antagonists in the same strips of guinea pig intestine are well correlated (11). Besides supporting the other evidence that binding *in vitro* involves sites that mediate opiate activity *in vivo*, these observations shed light on certain basic drug receptor concepts. Some receptor theories postulate that drugs can exert maximal pharmacological responses while occupying only a small fraction of the total number of pharmacologically active receptors. If a substantial portion of opiate receptors does not normally mediate pharmacological responses, and are thus "spare receptors," then there should be major discrepancies between drug concentrations filling a given percentage of binding sites and concentrations required to produce the same percentage of a maximal pharmacological response. Opiate concentrations occupying half the binding sites in the guinea pig intestine correspond closely, however, to those eliciting half-maximal pharmacological responses, whether agonist or antagonist, which suggests that opiate effects can be explained without invoking spare receptors.

Differential Receptor Interactions of Opiate Agonists and Antagonists

Opiate antagonists are important for several practical and theoretical reasons. Pure opiate antagonists, such as naloxone and naltrexone, are often life-saving antidotes to opiate overdose. Pure antagonists may also be valuable in treating heroin addicts. The notion is to administer a long-lasting pure antagonist, which will produce no analgesia and euphoria of itself, but which will prevent the effects of subsequently administered heroin. Thus deprived of its ability to elicit a "high," heroin will cease to be attractive to the addict.

Of greater theoretical and practical importance are opiates which are mixed agonist-antagonists, since some of these agents are relatively non-addicting analgesics. The idea of combining antagonist with agonist activity to lessen the addictive potential of an opiate stems from demonstrations that the opiate antagonist nalorphine possesses analgesic, and thus "agonist," properties (12). Unfortunately, nalorphine, cyclazocine, and levallorphan, antagonists "contaminated" with agonist activity, are not suitable for routine use in treatment of pain, because at analgesic doses they often produce anxiety, agitation, and psychotomimetic effects. Antagonists with yet more agonist activity, referred to here as mixed agonist-antagonists, have been more promising. Pentazocine, a mixed agonist-antagonist, is already clinically marketed and relieves pain with a low incidence of psychotomimetic effects

and with relatively little propensity to produce physical dependence and compulsive craving. Several other mixed agonist–antagonists, while not yet in routine clinical use, offer promise as relatively non-addicting analgesics.

Traditional opiate antagonists differ chemically from agonists only in the substitution of an *N*-allyl, *N*-cyclopropyl, or related group for the *N*-methyl of agonists. It is quite difficult to predict chemical features which result in a mixed agonist–antagonist. In our early studies of opiate receptor binding with buffer solutions lacking sodium, agonists and antagonists bound with similar affinity (3). Later, we discovered that low concentrations of sodium enhance receptor binding of antagonists but decrease the binding of agonists (13, 14). The effect of sodium is highly specific. It can be mimicked to some extent by lithium, whose atomic radius is similar to that of sodium, but not by other monovalent cations such as potassium, rubidium, or cesium. Sodium seems to accelerate the rate at which antagonists bind to the receptor (15) and to speed the dissociation of agonists from the receptor (14). To evaluate the influence of sodium on receptor interactions of a wide range of opiate agonists and antagonists, we measured the extent to which sodium alters the ability of drugs to inhibit receptor binding of [³H]naloxone. The resultant “sodium response ratio” is the ratio of the concentration of drug which inhibits [³H]naloxone binding 50% in the presence of sodium to the comparable concentration when sodium is omitted from the incubation medium. This sodium response ratio differentiates opiate agonists and antagonists. For pure antagonists, such as naloxone, the ratio is 1, while for a variety of opiate agonists, the ratio is between 12 and 60. Antagonists which are “contaminated” with some agonist activity have ratios somewhat greater than 1 but less than 3. The mixed agonist–antagonists we examined have ratios between 3.3 and 6.4, clearly in an intermediate area.

Thus it seems possible to predict the pharmacological properties of different opiates simply by measuring receptor interactions in the presence and absence of sodium, affording a rapid and inexpensive screen for mixed agonist–antagonist “non-addicting” analgesics. Chemists need not synthesize hundreds of grams of drug for screening in intact animals; a fraction of a milligram will suffice.

Model of Opiate Receptor Function

The selective and pharmacologically relevant influence of sodium on opiate receptor binding seems an integral feature of receptor functioning. Sodium seems to increase and decrease respectively the numbers of antagonist and agonist receptors. Since opiate agonists and antagonists are so similar chemically and compete with each other for receptor binding, we postulate that both drugs bind to the same receptor but that the opiate binding site can

vary as the receptor is transformed between two conformations (14, 16, 17). With normal body sodium levels, the receptor is largely in the "sodium" state for which antagonists have a selectively high affinity, whereas opiate agonist actions take place when agonists bind with their selectively high affinity for the "no-sodium" state. Antagonists block morphine actions by occupying sodium states of the receptor and shifting the equilibrium to reduce the number of no-sodium receptors available for morphine. Although agonists and antagonists have selective high affinity for no-sodium and sodium states of the receptor respectively, mixed agonist-antagonists would have similar affinities for the two forms.

The interconversion of the two forms of the opiate receptor presumably involves folding, unfolding, aggregation, disaggregation, or other modifications in protein structure, since the exquisite sensitivity of receptor binding to proteolytic enzymes (18) indicates that the opiate receptor is proteinaceous. One might expect protein-modifying reagents and enzymes that affect protein structure to alter the interconversion of receptor sites. Since the opiate receptor should normally be largely in the antagonist or sodium state, agents interfering with receptor interconversion should selectively reduce agonist binding. Consistent with these predictions is the observation that protein-modifying reagents and proteolytic enzymes in low concentrations do selectively reduce opiate agonist binding with negligible effects on antagonist binding (19, 20, 21). The effects of protein-modifying reagents and enzymes seem to be directly related to sodium binding by the opiate receptor, for the reagents and enzymes increase the sensitivity of opiate agonist binding to inhibition by sodium.

Divalent cations may also have a role in the normal functioning of the opiate receptor. Low physiological concentrations of manganese and magnesium affect the opiate receptor in a fashion diametrically opposite to sodium; they selectively increase the binding of opiate agonists, by reducing receptor sensitivity to sodium (22). Divalent cations act selectively; for while manganese, magnesium, and nickel are active, calcium fails to enhance opiate agonist binding. The influence of chelating agents indicates that endogenous divalent cations regulate opiate receptor functions. Thus, treating brain membranes with EDTA, which chelates most divalent cations, lowers opiate agonist binding, while EGTA, which chelates calcium but not manganese and magnesium, has no influence on receptor binding.

The postulated interconversion of agonist and antagonist conformations and the influence of sodium resemble general models for neurotransmitter receptor behavior (23, 24, 25). Normally receptor sites are in the antagonist or "off" conformation. Synaptic transmission occurs only when a compound binds selectively to the agonist conformation of the receptor. One or the other of the receptor states possesses a binding site for the ion whose con-

ductance is altered in synaptic transmission. When the neurotransmitter attaches to the receptor, the binding of this ion is altered to produce the appropriate change in ionic conductance. If the endogenous morphine-like substance is a neurotransmitter, one would speculate that sodium is the ion whose conductance is changed by synaptic activity.

Direct evidence for selective interactions of an ion and receptor binding in synaptic transmission has been obtained for the influence of chloride on glycine receptor binding. Receptor sites for glycine, a major inhibitory neurotransmitter in the mammalian central nervous system, can be labeled with radioactive strychnine, a potent glycine antagonist (26). Chloride is the ion whose conductance is increased by the inhibitory synaptic actions of glycine. Chloride and other anions inhibit the binding of strychnine to the glycine receptor in proportion to their ability to mimic the synaptic activities of chloride (27).

Regional Mapping

Brain functions whose distributions parallel those of opiate receptors may be implicated in opiate actions. Because opiates elicit analgesia, brain structures mediating pain are natural suspects. Sharp, prickly pain, which can be discretely localized, is conveyed by a laterally located sensory pathway with somatotopic representation in the ventrobasal thalamus among other regions. Slower, more chronic and poorly localized burning pain involves multisynaptic, slowly conducting, medially located pathways, especially the paleospinothalamic and spinoreticulodiencephalic pathways. These lack somatotopic representation and include areas of the brain such as the periaqueductal gray, the medial nuclei of the thalamus, and several parts of the limbic system (28).

Opiate receptor binding varies dramatically throughout the monkey and human brain (29, 30). The amygdala binds most, but only slightly more than the periaqueductal gray of the midbrain, hypothalamus, and medial thalamus. Receptor binding in the caudate nucleus is also high. Within the cerebral cortex there are marked variations: the frontal cortex for example binds more than four times the amount of opiate bound by the precentral, postcentral gyri, and occipital pole. Receptor binding is very low in the spinal cord, cerebellum, and white matter.

This map closely resembles the distribution of paleospinothalamic and spinoreticulodiencephalic pain pathways, and certain of these areas, especially the periaqueductal gray, correspond to those in which implantation of morphine most effectively produces analgesia (31). Opiate antagonists elicit withdrawal symptoms in addicted animals best when implanted in the medial thalamus, which is also very rich in opiate receptors (32). The amygdala,

apparently an exception not classically associated with pain pathways, may relate to affective components of pain since electrical stimulation of the amygdala does produce fight and flight reactions. Euphoric actions of morphine may be mediated by the amygdala and other areas of the emotion-regulating limbic system rich in opiate receptors.

Recent autoradiographic studies provide even more precise localization of opiate receptor sites (33). For such studies it was first necessary for us to achieve reliable techniques for labeling receptors in the intact animals (34). After intravenous administration of [^3H]diprenorphine, an extremely potent antagonist, 80% or more of radioactivity accumulating in the brain is associated with opiate receptor sites (33). For successful autoradiography, diffusion of the radioactive drug away from the receptor site during the fixation process must be prevented, a difficult task with a drug such as an opiate which does not form covalent chemical linkages to tissue proteins during fixation. In our studies, fixing tissue at very low temperature prevents leakage of [^3H]diprenorphine from receptor sites so that essentially all autoradiographic grains are associated with the opiate receptor (33). Grain density parallels the regional distribution of the opiate receptor as determined by dissection and biochemical assays. At a microscopic level, opiate receptor binding is even more discretely localized (Table 4-1). At one level of the midbrain, grains are highly concentrated over the locus coeruleus with much lower grain counts in closely adjacent nuclei. Interestingly, small doses of morphine slow firing rates of locus coeruleus cells but not adjacent cells (35). Since the locus coeruleus consists almost exclusively of noradrenalin cell bodies, its high density of opiate receptors may explain the numerous reports of effects of opiates on the metabolism of biogenic amines including noradrenalin (36). At another level of the midbrain, grains concentrate over the zona compacta of the substantia nigra, whereas the adjacent zona reticulata of the substantia nigra has very few receptor grains. The zona compacta but not the zona reticulata consists exclusively of dopamine cell bodies. Thus both major catecholamine systems on the brain are intimately related to opiate receptor sites. An association with major sensory systems in the central nervous system is indicated by the sharp band of opiate receptor grains overlying the substantia gelatinosa in the lower brain stem and spinal cord (33). The substantia gelatinosa is an important "way station" for the upward conduction of sensory information relating to pain.

Mapping opiate receptor sites by autoradiography is still in a preliminary stage. It is however already clear that this information will help greatly in elucidating the physiological and pharmacological roles of opiate receptor sites. Neurophysiologists who would like to record from cells containing opiate receptor sites ought to focus on the locus coeruleus, the zona compacta of the substantia nigra, and other areas enriched in these receptors.

TABLE 4-1. Distribution of autoradiographic grains labeling the opiate receptor in coronal sections of rat brain

| Region | Autoradiographic grains per 100 μm^2 |
|---|--|
| Nucleus caudatus putamen | |
| “Streak” ventral to corpus callosum | 11.5 |
| “Clusters” | 10.5 |
| Low density areas | 1.7 |
| Fibers | 1.2 |
| Substantia gelatinosa (spinal cord) | 10.4 |
| Locus coeruleus | 10.0 |
| Amygdala medialis | 6.5 |
| Amygdala centralis | 5.8 |
| Zona compacta of substantia nigra | 6.5 |
| Thalamus medialis | 5.5 |
| Periventricular substance | 5.2 |
| Habenula | 5.1 |
| Lateral habenula | 5.0 |
| Nucleus periventricularis (of hypothalamus) | 3.8 |
| Nucleus ventromedialis (of hypothalamus) | 2.8 |
| Dentate gyrus | 2.1 |
| Motor cortex | 2.0 |
| Zona reticulata of substantia nigra | 1.9 |
| Hippocampus | 1.7 |
| Corpus callosum | 0.7 |
| Fimbria | 0.8 |
| Ventricle III | 1.3 |
| Pyriiform cortex | 1.1 |
| Cerebellum | 0.8 |
| Nucleus of cranial nerve V | 0.8 |

Note. These data are derived from the study of Pert, Kuhar, and Snyder (33). Rats (170 g) were injected with 125 μCi of [^3H]diprenorphine (13 Ci mmol^{-1}), killed at 1 h and brains rapidly cut into 3–4 mm coronal sections, placed on microtome chucks and lowered slowly into liquid nitrogen “slush.” Sections of 4 μm thickness were cut at -18°C in a Harris cryostat microtome and transferred by “thaw-mounting” in the dark to slides already coated with Kodak-NTB-3 emulsion. After 5 weeks of exposure at low humidity (4°C) in a dark lead-lined cabinet, slides were developed at 17°C in Dektol for 2 min. Development was terminated by Kodak Liquid Hardener and slides were fixed in hypo (Kodak). After washing in running tap water, sections were stained with pyronine Y, dried, mounted with Permout, and viewed with a Zeiss Universal microscope. Control slides prepared for positive and negative chemography showed no significant fading of latent images or spurious generation of grains after 60 d of exposure. Grain counts are the means of 2,400 μm^2 areas.

The Endogenous Morphine-like Factor

Man was not made with morphine in him. Is the opiate receptor “designed” for a normally occurring morphine-like substance, perhaps a neurotransmitter? If so, such a system is widespread among animals. Opiate receptor binding with almost identical substrate specificity has been detected in the central nervous system of all vertebrates, and the most primitive fish have as many opiate receptors as mammals including man (37). Strikingly, no opiate receptor binding can be demonstrated in any invertebrates, which may indicate a relationship of the opiate receptor to known differences in patterns of vertebrate and invertebrate neuronal connections. The possibility that the opiate receptor is associated with a neurotransmitter system is strengthened by sub-cellular fractionation studies which indicate that in mammalian brain homogenates opiate receptor binding is most enriched in synaptosome fractions containing nerve endings with their associated postsynaptic membranes (37). When these are subjected to hypotonic lysis, opiate receptor binding is recovered primarily in the synaptic membrane fraction, as would be expected for a neurotransmitter receptor (38). A direct approach to identifying a possible neurotransmitter associated with the opiate receptor was adopted by Hughes (39), who found in brain extracts a substance which mimics the ability of morphine to inhibit electrically induced contractions of smooth muscle preparations such as the mouse vas deferens or guinea pig intestine, while Terenius (40) and Pasternak and Snyder (41) reported that brain extracts compete for opiate receptor binding. The substance studied by both approaches seems to be the same or very similar. The morphine-like factor or “enkephalin” is a peptide, degraded by carboxypeptidase A and B as well as leucine-aminopeptidase and to a lesser extent by chymotrypsin but not by trypsin (39, 41, 42). In opiate binding assays, the material behaves like an agonist, since its ability to compete for binding is impaired by sodium and protein modifying reagents but enhanced by manganese (41). Its regional distribution in calf, rat, and rabbit brain is closely similar to that of the opiate receptor itself (Table 4-2) (39, 40, 41, 42). Preliminary chemical analysis indicates that the morphine-like factor contains three residues of glycine and one each of phenylalanine, tyrosine, and methionine and may also contain tryptophan (39, 42). A chemically different peptide, which also mimics the effect of morphine on smooth muscle, has been isolated from pituitary glands (43).

If the morphine-like factor is a neurotransmitter, how might it affect postsynaptic cells? Morphine and other opiates applied iontophoretically to single cerebral cortex cells inhibit firing in proportion to their pharmacological potency with marked stereospecific actions and antagonism by naloxone, suggesting that the morphine-like factor may be an inhibitory transmitter (44). Cells of rats addicted to morphine are no longer inhibited

TABLE 4-2. Regional localization of the morphine-like factor and opiate receptors in bovine and rat brain

| | Morphine-like factor (U per mg protein) | Opiate receptor binding (cpm per 0.1 mg protein) |
|----------------------------|--|---|
| Bovine | | |
| Caudate | 480 | 320 |
| Hypothalamus | 250 | 282 |
| Spinal cord | 140 | 205 |
| Pons | 135 | 231 |
| Cerebral cortex (parietal) | 80 | 173 |
| Thalamus | 75 | 179 |
| Cerebellum | 50 | 86 |
| Medulla oblongata | 50 | 88 |
| Corpus callosum | 10 | 61 |
| Rat | | |
| Caudate | 480 | 900 |
| Brain stem (midbrain) | 140 | 220 |
| Cerebral cortex | 80 | 210 |
| Cerebellum | None | None |

Note. Data are derived from the study of Pasternak and Snyder (41). In assays for the morphine-like factor, brains were homogenized in ten volumes of 0.32 M sucrose with a Potter-Elvehjem homogenizer, and centrifuged at 100,000g for 1 h. The pellet was resuspended in 2 volumes of 10 mM Tris-HCl buffer (pH 7.7 at 25°C) immersed in a bath of boiling water for 15 min, recentrifuged at 100,000g for 1 h, and the supernatant assayed by adding 50–200 µL to an opiate receptor binding assay with [³H]naloxone and 1 mM MnCl₂. Opiate receptor assays were performed as previously described (20). The experiments were replicated four times with less than 20% variation between experiments.

by administered morphine and may even show a paradoxical excitation. Because these changes with addiction occur at the opiate responsive cell itself, the locus of the fundamental alteration in opiate addiction would seem to be at the level of the cell membrane possessing the opiate receptor.

Possible Mechanisms of Addiction

The term “addiction” is not easily defined. Most people consider opiate addiction to comprise three major elements: tolerance, physical dependence, and compulsive craving. Tolerance means simply that, after prolonged administration of a drug, the organism can “tolerate” more of it, that is, it is now less sensitive to the drug than before. Physical dependence refers to the

fact that when the drug is withdrawn or when the animal or person is treated with an opiate antagonist, withdrawal symptoms become evident which are usually in a direction opposite to the initial effects of the drug. Thus whereas morphine produces pupillary constriction, constipation, and sedation, during withdrawal the pupils are dilated and there is diarrhea and central excitation. Compulsive craving is very difficult to evaluate; it refers to the addict's propensity for the drug even long periods after he has been physically withdrawn. Just as tolerance can be looked on as a state of decreased sensitivity to opiate agonists, so in physical dependence animals and humans become more sensitive to antagonists. Withdrawal symptoms can be elicited by much lower doses of antagonists in severely than in mildly addicted animals.

There are two types of model which explain opiate addiction; one of them involves a change at the level of the opiate receptor or closely allied structures, whereas the other requires no such alterations (45, 46, 47, 48, 49). In the latter model, one presumes that morphine suppresses some neuronal system in the brain. To compensate, a completely separate system increases its activity to counteract the suppressive action of morphine; whereupon the organism is "tolerant." When morphine is withdrawn, the "overactive" second system produces withdrawal symptoms.

Hypersensitivity to antagonists coupled with subsensitivity to agonists in addiction is reminiscent of the properties of the sodium or "antagonist" state of the opiate receptor as contrasted to the no-sodium or "agonist" state. It may be speculated that tolerance and physical dependence reflect a change in the opiate receptor so that it is less capable of assuming the agonist form, favoring instead the antagonist, sodium form. If this were so, addicted animals should be "subsensitive" to opiate agonists, since the receptor would be less frequently in the "agonist" state to mediate drug effects. Similarly, the organism would be supersensitive to antagonists because of the predominant sodium or antagonist state of the receptor. Though opiate receptor assays both *in vitro* and *in vivo* have failed to show systematic changes related to the addicted state (13, 50), it is conceivable that such a subtle alteration may be reflected only in membrane properties which occur secondarily to the binding itself. In this connection, recent studies of opiate effects on a neuroblastoma-glioma hybrid in cell culture are illuminating. Opiate agonists, in proportion to their affinity for opiate receptor binding sites in this clone, decrease adenylate cyclase (51, 52, 53) and enhance the accumulation of cyclic GMP (53). Opiates also reverse the stimulation of adenylate cyclase by prostaglandin E_1 and adenine (51). Cells exposed chronically to morphine become "tolerant" to the effects of morphine so that previously active doses no longer antagonize the prostaglandin stimulation of adenylate cyclase (54). When the cells are put into "withdrawal" by naloxone, they become "super-

sensitive" to the ability of prostaglandin E_1 to stimulate adenylate cyclase, an effect opposite to that of morphine itself in drug-naïve cells (54).

Of course, one might argue that the cyclic nucleotide changes are relevant only to these cancerous cells of the nervous system in tissue culture. Previously, Collier and Roy (55) had described a prostaglandin-stimulated adenylate cyclase in mammalian brain which is inhibited by morphine and other opiates, and may be related to the neuroblastoma-glioma effects. Moreover phosphodiesterase inhibitors, which elevate brain cyclic AMP levels, elicit in rats behavioral changes resembling the opiate withdrawal syndrome and which are enhanced by as little as 0.03 mg/kg^{-1} of naloxone (56, 57).

In summary, recent identification of opiate receptor sites in the central nervous system by biochemical means has spurred a great body of research directed both at pharmacological actions of opiates as well as at the normal functioning of the opiate receptor. An apparently novel peptide neurotransmitter, the morphine-like factor, is being characterized.

As is the case with numerous hormones and neurotransmitters, this morphine-like factor may have as its second messenger cyclic nucleotides, either cyclic AMP or cyclic GMP. Most dramatically, it seems possible that changes in the adenylate cyclase associated with the opiate receptor and conceivably in the opiate receptor itself may begin to answer the many riddles of addiction.

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CHAPTER 5

Morphine-Like Peptides in Mammalian Brain

Isolation, Structure Elucidation, and Interactions With the Opiate Receptor

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Specific opiate receptor binding (1, 2, 3) portended the existence of a “natural” opiate “enkephalin” that was demonstrated in brain by its effects on smooth muscle (4, 5) and by its competition for receptor binding (6, 7, 8, 9), whereas pituitary extracts contain a different opiate-like substance (10). Regional (4, 8, 9, 11, 12) and subcellular (13) localizations of enkephalin parallel opiate receptor binding. Hughes et al. (14) identified pig brain enkephalin as two pentapeptides, Tyr-Gly Gly Phe-Met (methionine enkephalin, Met-enkephalin) and Tyr-Gly Gly Phe-Leu (leucine enkephalin, Leu-enkephalin), with about four times more Met-enkephalin than Leu-enkephalin. In bovine brain we independently isolated and identified the same two peptides with four times more Leu-enkephalin than Met-enkephalin (15), here described in detail.

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Materials and Methods

Extraction and Purification of Enkephalin

Fresh calf brains minus cerebella, homogenized by Polytron (Brinkmann) in 2–5 volumes of 10 mM Tris HCl buffer (pH 7.7 at 25°), were centrifuged at 100,000g for 60 min, and the supernatant was heated for 15 min in a boiling-water bath. The boiled homogenate was centrifuged at 100,000g for 60 min and the supernatant was lyophilized. The powder was extracted in methanol (20 vol/wt) and the methanol was evaporated at 30°. The residue, dissolved in 20 mL of 50 mM Tris-HCl buffer (pH 7.7 at 25°), was applied to a Biogel P₂ column (95×3.1 cm) that was prepared and eluted in the same buffer. Fractions 21–29 were pooled, lyophilized, and resuspended in 20 mL of 100 mM NH₄-acetate buffer, pH 7.4, applied to a column of AG-1-X2 (200–400 mesh, Bio-Rad, 35×1.8 cm), and prepared and washed with 150 mL of the NH₄-acetate buffer. The column was eluted with a linear pH gradient of 250 mL of 100 mM NH₄-acetate buffer, pH 7.4, and 250 mL of 100 mM acetic acid. Fractions (7 mL) with peak opioid activity (eluted at pH 5.6–5.9) were pooled, lyophilized, suspended in 10 mM HCl, and applied to a column of AG-50W-X2 (200–400 mesh, Bio-Rad, 25×1.1 cm) prepared in 10 mM HCl. Fractions were eluted, first successively with 100 mL of 10 mM HCl and 100 mL of 100 mM NH₄-acetate buffer, pH 7.4, and then with a linear pH gradient of 250 mL of 100 mM NH₄-acetate buffer, pH 7.4, and 250 mL of 100 mM NH₄OH. Five-milliliter fractions were lyophilized, suspended in 0.5 mL of H₂O, and assayed for opiate receptor activity. The peak fractions were applied to an Amberlite CG-400 column (26×1.1 cm), prepared in 200 mM NH₄-formate buffer, pH 8.5, washed with 100 mL of 200 mM NH₄-formate buffer, pH 8.5, and then eluted with a linear pH gradient of 200 mL of 0.2 M NH₄-formate buffer (pH 8.5) and 200 mL of 0.2 M formic acid. After lyophilization, peak fractions (10–50 µL) were applied to 3 MM Whatman paper and subjected to high-voltage paper electrophoresis at 30 V/cm for 90 min at pH 2 (8% acetic acid, 2% formic acid). Material was extracted from strips in 2.5 mL of 10% acetic acid. The extracts were lyophilized, suspended in 0.5 mL of H₂O, and assayed for enkephalin activity. Eluates of the Biogel P₂, the AG-1-X2, and the AG-50W-X2 columns were monitored for “protein” by UV absorbance at 280 nm. Samples from Amberlite CG-400 and the paper electrophoresis steps were assayed by the fluorescamine method (16) with glycine as a standard.

Determination of Amino Acid Composition and Sequence

Two nanomoles of enkephalin, purified through the electrophoresis step (fluorescamine, glycine equivalents), were hydrolyzed with 5.5 M constant boiling HCl (Pierce Co.) for 2–18 hr at 105°. Amino acid composition of the

hydrolysate was determined after dansylation by chromatography on Cheng-Chin polyamine layer sheets (17) with 18 standard dansylated amino acids (Pierce Co.). For determination of tryptophan, samples were hydrolyzed with 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (18) or with 3 M mercaptoethanesulfonic acid (19) at 110° for 48 and 72 hr.

The amino acid sequence was investigated by the sequential degradation dansyl-Edman procedure (20). The carboxyl-terminal was determined by incubating 10 nmol of enkephalin for 0–15 min in H₂O at 25° on a 0.5×3 cm column of carboxypeptidase A bound to Sepharose (Worthington Biochemical Co.). The eluate (10 mL) from the carboxypeptidase column was lyophilized and dansylated, and amino acids were determined by chromatography on polyamine sheets. Relative amounts of the dansylated amino acids were quantified by comparison to 0.01–1.0 nmol of standard dansylated amino acids. The polyamine sheets (7.5×7.5 cm) were placed in two solvents: (i) H₂O–90% formic acid (200:3, vol/vol), and (ii) benzene-glacial acetic acid (0:1, vol/vol). A third solvent, *n*-heptane–butanol–glacial acetic acid (3:3:1, vol/vol/vol), gave similar results. Synthetic peptides Tyr-Gly Gly Phe-Leu and Tyr-Gly Gly Phe-Met were a generous gift of Dr. D. Hauser and Dr. F. Cardinaux, Sandoz, Ltd., Switzerland.

Opioid activity was assayed as the ability to inhibit specific [³H]naloxone (New England Nuclear Corp., 20 Ci/mmol) binding to rat brain membranes (21) as previously described (15). One unit of opioid activity “enkephalin” was defined as that amount that yields 50% occupancy in a 200-μL assay, according to Colquhoun (22).

Results

Purification and Isolation of Enkephalin

We isolated apparently homogeneous enkephalin by a six-step purification. Almost all activity is extracted into absolute methanol, with a 4.6-fold purification (Table 5–1). Biogel P₂ columns give a further 7-fold purification. The AG-1-X2, AG-50W-X2, and Amberlite-CG-400 ion exchange columns provide successive enrichments of 13-fold, 12-fold, and 5-fold (Figures 5–1 and 5–2; Table 5–1). Activity elutes from the Amberlite-CG-400 column in a single peak at pH 5.6–5.9 (Figure 5–2), and subsequent high-voltage paper electrophoresis at pH 2 resolves enkephalin from other fluorescamine-reactive material, yielding apparently homogeneous enkephalin.

Assessment of Enkephalin Purity and Determination of Amino Acid Composition and Sequence

In Biogel P₂ chromatography, enkephalin purified through the electrophoresis step elutes as a single peak corresponding to the only peak of fluoresca-

TABLE 5-1. Purification of calf brain enkephalin

| Step of purification | Enkephalin activity (unit/nmol of glycine equivalent) | Purification (-fold) |
|---|---|----------------------|
| 1. Brain extract | 0.006 | — |
| 2. Methanol extraction | 0.028 | 4.6 |
| 3. Biogel-P ₂ gel chromatography | 0.21 | 34.6 |
| 4. AG-1-X2 ion exchange | 2.6 | 435 |
| 5. AG-50W-X2 ion exchange | 30 | 5,019 |
| 6. Amberlite-CG-400 ion exchange | 155 | 25,900 |
| 7. High-voltage paper electrophoresis, pH 2.0 | 292 | 48,600 |

Note. Enkephalin activity was assayed as in *Materials and Methods*. The purification scheme was repeated three times, with less than 20% variation in extent of purification in all steps.

mine-reactive material, reflecting an apparent molecular weight of about 1,000 (Figure 5-3). High-voltage paper electrophoresis at pH 2 and pH 6.5 reveals single peaks of fluorescamine-reactivity and enkephalin activity. At pH 2, enkephalin activity migrates toward the anode slightly behind aspartic acid, indicating an approximate molecular weight of 600-800 (23), like pig brain enkephalin (5, 14). At pH 6.5, bovine brain enkephalin activity migrates very slightly toward the anode, like glycine and valine, so at this pH enkephalin lacks a strong charge (Figure 5-3).

After dansylation and HCl hydrolysis a single spot on polyamine sheets corresponds to *bis*-tyrosine, indicating a single NH₂-terminal amino acid, tyrosine. Hydrolysis after dansylation with 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole reveals no tryptophan.

Amino acid composition, assessed by dansylation after HCl hydrolysis and polyamine sheet chromatography, shows glycine, tyrosine, phenylalanine, leucine, and methionine. Spots for tyrosine, phenylalanine, and leucine are of equal intensity, whereas the spot for methionine is substantially weaker and that for glycine is more intense. Total hydrolysis with 3 M mercaptoethanesulfonic acid reveals no tryptophan.

The amino acid sequence was determined by dansyl-Edman sequence determination. The NH₂-terminal amino acid is confirmed as tyrosine, and the second, third, and fourth amino acids correspond to glycine, glycine, and phenylalanine, respectively. In repeated experiments the fifth amino acid in the sequence appears to consist of both leucine and methionine, with the leucine spot about four times more intense than the methionine spot. Further treatment reveals no additional amino acids, suggesting that enkephalin

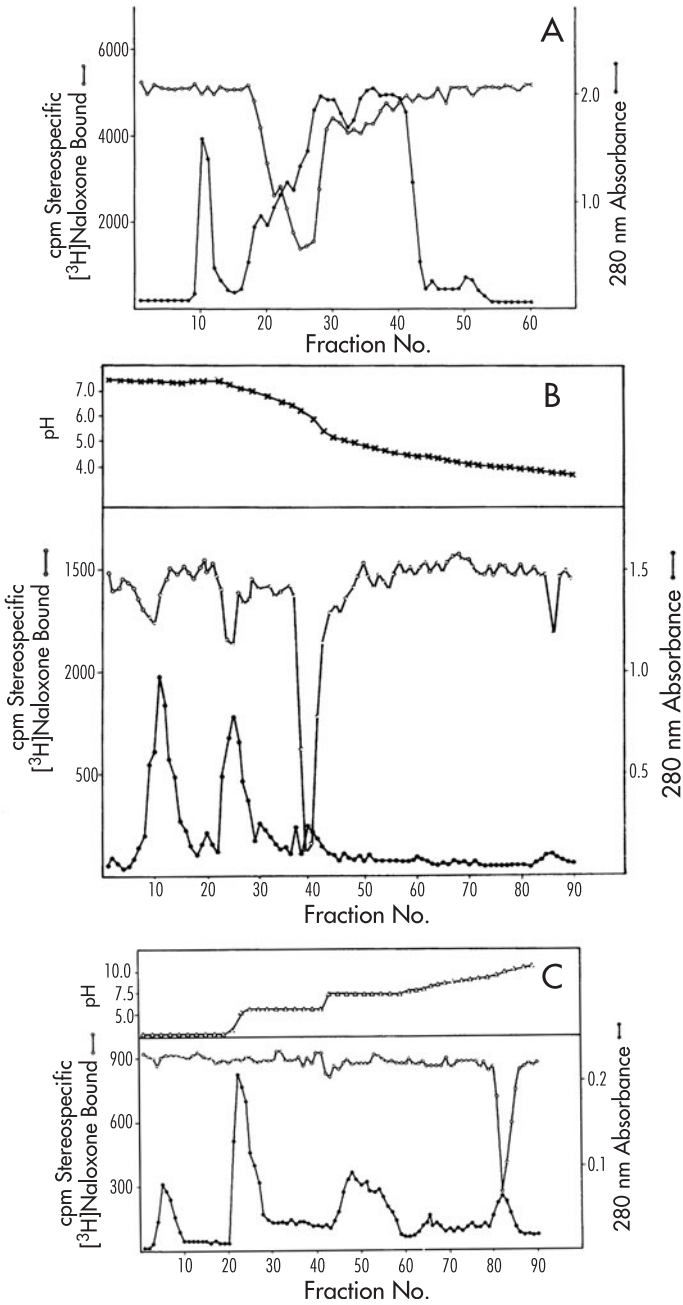


FIGURE 5-1. Purification of enkephalin.

(A) Biogel P_2 gel chromatography. (B) AG-1-X2 ion-exchange chromatography. (C) AG-50W-X2 ion-exchange chromatography.

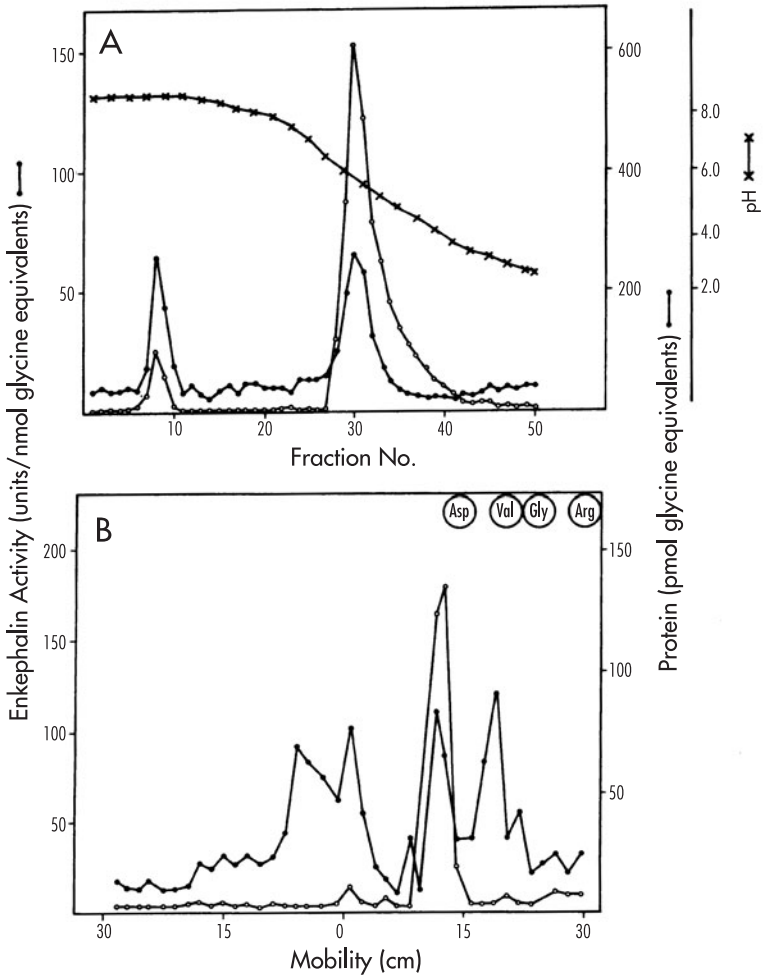


FIGURE 5-2. Purification of enkephalin.

(A) Amberlite CG-400 ion-exchange chromatography. (B) High-voltage paper electrophoresis.

may consist of two pentapeptides whose respective carboxyl terminals are leucine and methionine. Carboxyl-terminal determination by incubation with carboxypeptidase A bound to Sepharose for 5 or 8 min reveals a mixture of leucine and methionine, with about four times more leucine than methionine. If leucine were the carboxyl terminal and methionine were the next amino acid, further treatment with carboxypeptidase A should reveal an increase in methionine. However, incubation for 15 min fails to alter the relative amounts of leucine and methionine but reveals, in addition, phenyl-

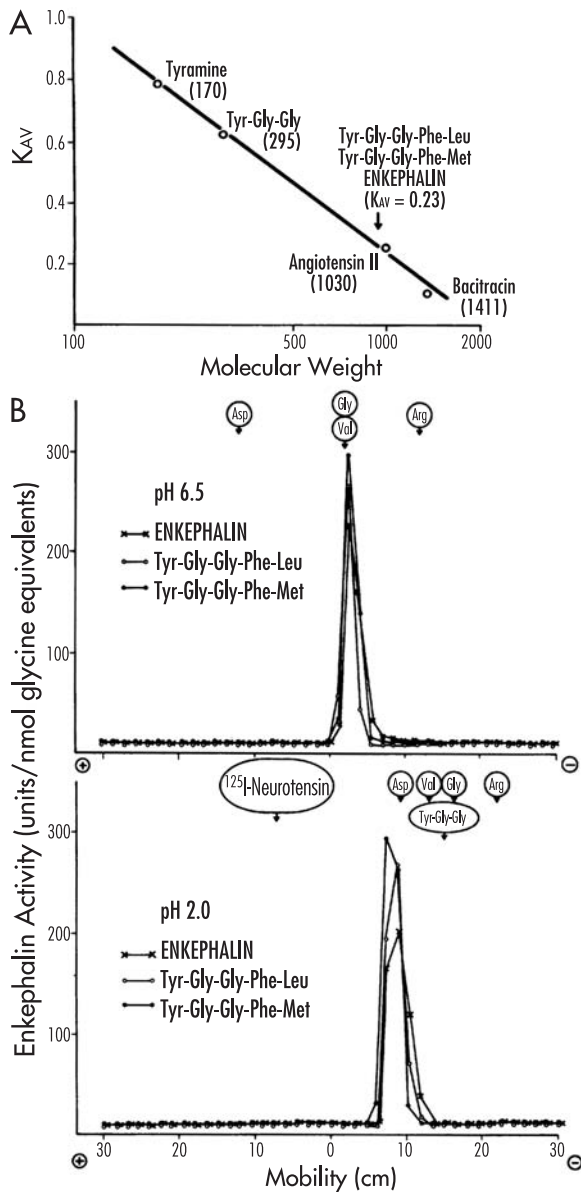


FIGURE 5-3. Biogel P₂ chromatography and high-voltage paper electrophoresis of enkephalin and the synthetic peptides, Tyr-Gly Gly Phe-Leu and Tyr-Gly Gly Phe-Met.

(A) Purified and synthetic enkephalins were chromatographed as reported (11).
(B) Purified and synthetic enkephalins were subjected to electrophoresis as in *Materials and Methods*.

alanine in concentrations corresponding to those of leucine. These observations support the suggestions derived from the dansyl-Edman technique that there are two peptides whose respective carboxyl terminals are leucine and methionine. Bovine brain enkephalin activity is therefore attributable to two pentapeptides whose respective amino acid sequences are Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, identical to sequences of pig brain enkephalins (14). However, whereas pig brain contains three to four times more Met-enkephalin than Leu-enkephalin, bovine brain possesses about four times more Leu-enkephalin than Met-enkephalin.

In Biogel P₂ chromatography, natural enkephalin elutes at an identical position as the synthetic Leu-enkephalin and Met-enkephalin (Figure 5–3). In high-voltage paper electrophoresis at pH values of 2.0 and 6.5, natural enkephalin migrates as a single sharp peak, identical to those of synthetic Leu-enkephalin and Met-enkephalin (Figure 5–3).

Interactions of Natural and Synthetic Enkephalins With the Opiate Receptor

Hughes et al. (14) found Met-enkephalin about three times as potent as morphine in inhibiting [³H]naloxone binding in sodium-free homogenates of guinea pig brain. In the absence of sodium, native enkephalin inhibits [³H]naloxone binding with an IC₅₀ (median inhibitory concentration) of 24 nM, the same as Leu-enkephalin, whereas Met-enkephalin is about three to four times more potent (Table 5–2). Even at 1 μM, the tripeptide Tyr-Gly-Gly fails to alter [³H]naloxone binding.

Sodium but not potassium decreases receptor binding of opiate agonists while enhancing binding of antagonists (24), whereas manganese but not calcium enhances agonist but not antagonist binding (25). At 10 mM NaCl natural and synthetic enkephalins become less potent in competing for [³H]naloxone binding, an agonist profile, whereas competition by nonradioactive naloxone is unaffected. Potassium fails to alter the potencies of natural and synthetic enkephalins and morphine. With 0.25 mM manganese, morphine and synthetic and natural enkephalins become more potent in inhibiting [³H]naloxone binding, whereas calcium is ineffective. Sodium diminishes potencies of natural enkephalin and synthetic Leu-enkephalin six to seven times, whereas manganese enhances potency 4-fold. By contrast, Met-enkephalin is reduced in potency only 3.75-fold by sodium and is enhanced in potency 3.0-fold by manganese. The closely similar effect of these ions upon natural enkephalin and Leu-enkephalin is consistent with our observation that about 80% of natural enkephalin consists of Leu-enkephalin. The more marked influences of sodium on receptor interactions of leucine than of methionine enkephalin suggest that Leu-enkephalin is a “purer” agonist than Met-enkephalin.

TABLE 5-2. Inhibition of [³H]naloxone binding by enkephalin: ionic effects

| Ions | IC ₅₀ (nM) ^a | | | | |
|---------------------------|------------------------------------|-------------------------|-------------------------|----------|----------|
| | Natural enkephalin | Tyr-Gly-Gly- Phe-Leu | Tyr-Gly-Gly- Phe-Met | Morphine | Naloxone |
| None | 25 | 30 | 8 | 5 | 3 |
| NaCl | 150 | 200 | 30 | 30 | 3 |
| KCl | 27 | 32 | 9 | 5 | 3 |
| MnCl ₂ | 6 | 8 | 2.7 | 2 | 3 |
| CaCl ₂ | 23 | 30 | 7 | 2 | 3 |
| Sodium shift ^b | 6.0 | 6.7 | 3.75 | 6.0 | 1.0 |
| Manganese shift | 0.24 | 0.27 | 0.34 | 0.4 | 1.0 |

Note. Ion concentrations were 100 mM for NaCl and KCl and 0.25 mM for MnCl₂ and CaCl₂.

^aIC₅₀ is concentration that inhibits [³H]naloxone binding 50%.

^bSodium and manganese shifts, respectively, are the ratio of IC₅₀ with NaCl to IC₅₀ in its absence or the ratio of IC₅₀ with manganese to IC₅₀ in the absence of MnCl₂.

Discussion

Guillemin et al. (26) identified a hexadecapeptide “α-endorphine” in pig neurohypophysis-hypothalamus incorporating the Met-enkephalin sequence and with guinea pig ileum potency resembling Met-enkephalin. Alpha-endorphine corresponds to fragments 61–76 of the pituitary peptide β-lipotropin (27, 28, 29).

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CHAPTER 6

Opioid Peptide Enkephalin

Immunohistochemical Mapping in Rat Central Nervous System

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The opiate-like pentapeptides methionine-enkephalin (Met-enk) and leucine-enkephalin (Leu-enk) (1, 2) appear to be endogenous ligands for the opiate receptor. The regional localization of enkephalin in mammalian brain, determined biochemically, resembles that of opiate receptor binding (3, 4, 5, 6). In subcellular fractionation studies, enkephalin is localized to synaptosomal fractions that contain nerve terminals (7). Autoradiographic studies of the opiate receptor reveal sharply defined localizations to structures mediating functions affected by opiates, such as pain perception (8, 9, 10). If the enkephalins are neurotransmitters or neuromodulators associated with opiate receptors, one might expect enkephalin to be localized microscopically to neuronal systems impinging on opiate receptors. Preliminary immunohistochemical studies show immunoreactive enkephalin fluores-

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cence in nerve fibers and terminals with highest densities in areas enriched in opiate receptors (11). We now report a detailed mapping of the rat central nervous system for immunoreactive enkephalin.

Materials and Methods

Antisera Preparation

Met-enk or Leu-enk (20 mg) was coupled to keyhole limpet hemocyanin (10 mg) by incubation for 30 min in distilled water at room temperature with 150 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The material was dialyzed extensively against distilled water, lyophilized, suspended in 3 mL of distilled water, and stored. Guinea pigs were immunized with 1 mg of the hemocyanin-coupled enkephalin diluted 1:10 in saline and mixed 1:1 with Freund's complete adjuvant. Rabbits were injected with 1.6 mg of this conjugate. The immunization was repeated three to four times at 3 to 4 week intervals using incomplete Freund's adjuvant. The guinea pigs and rabbits were bled 7–10 days after the third and subsequent immunizations and the sera were tested for enkephalin binding in radioimmunoassays (12; Simantov, Childers, and Snyder, unpublished data). Enkephalin binding by these antisera was not displaced by high concentrations of substance P, glucagon, insulin, neurotensin, angiotensin II, acetylcholine, norepinephrine, dopamine, γ -aminobutyric acid, 3':5'-cyclic AMP, or 3':5' cyclic GMP (12). Enkephalins were more than 200 times as potent as α -endorphin and β -endorphin in displacing enkephalin binding to guinea pig or rabbit antisera against enkephalin. Met-enk was 6–8 times more potent than Leu-enk in displacing [3 H]Met-enk binding to guinea pig and rabbit antisera against Met-enk. Leu-enk was 8–10 times and 1,000 times more potent than Met-enk in displacing [3 H]Leu-enk binding to guinea pig and rabbit antisera against Leu-enk, respectively. However, all antibodies could bind both enkephalins.

Immunofluorescence

Sprague-Dawley rats (150–200 g) were perfused through the aorta for 20 min with cold 4% depolymerized paraformaldehyde in 0.05 M sodium phosphate buffer, pH 7.3; the brains were dissected into four blocks, postfixed for 3 hr, and then stored at 4° in 0.24 M sucrose/0.05 M sodium phosphate buffer, pH 7.3. Sections of 12–16 μ m were cut at -10° with a Harris cryostat and stored at -20° for convenience until stained. No change in patterns of specific fluorescence has been noted after storage for up to 6 weeks. For staining, the sections were incubated for 30 min at 37° with 1:10–1:100 dilutions of rabbit or guinea pig antisera to Met-enk or Leu-enk diluted in 0.05 M phosphate-buffered saline, pH 7.4, containing 0.1–0.2% Triton X-100. The sections were washed three times (5 min each) with the same phosphate buffer con-

taining 0.05% Triton X-100 and then incubated for 15 min at 37° with fluorescein-conjugated goat antibody against guinea pig IgG or fluorescein-conjugated goat antibody against rabbit IgG (IgG fraction, Cappel Laboratories) diluted 1:50 with phosphate buffer containing 0.05–0.1% Triton X-100. The sections were then washed three times (5 min each) in phosphate buffer containing 0.2% Triton X-100, dipped in H₂O, mounted with 0.05 M sodium bicarbonate buffer, pH 8.4, diluted 1:1 with glycerol, and examined with a Zeiss Universal fluorescence microscope.

Results

Enkephalin immunoreactivity varies markedly among different structures in the central nervous system. Regional variations parallel variations in opiate receptor density determined either by autoradiography (Figures 6–1, 6–2, 6–3, 6–4) (8, 9, 10) or biochemical assays (13, 14, 15) and also parallel the regional distribution of endogenous enkephalin measured either by radioimmunoassay (12) or radio-receptor assay (3, 4, 5, 6). Control experiments utilizing preimmune serum or serum previously incubated with Met-enk or Leu-enk (Figure 6–1) show negligible fluorescence.

Throughout the rat central nervous system enkephalin-like immunofluorescence is localized to fiber-like structures and small swellings that resemble biogenic amine-containing varicosities. This suggests a localization to fibers and nerve terminals. In some areas, such as the cerebral cortex, certain hypothalamic nuclei, lateral reticular formation, globus pallidus, and spinal cord, fluorescence is localized in nonnuclear portions of neuronal perikarya. Immunofluorescent patterns appear similar whether rabbit or guinea pig antisera to Met-enk or Leu-enk are utilized.

In the upper cervical spinal cord, a dense band of dot and fiberlike fluorescence is confined to laminae I and II (Figures 6–1D and 6–3), resembling opiate receptor localization. Fluorescent fibers occur in the dorsal white matter adjacent to the lateral borders of the dorsal gray matter, within the ventral gray matter (Figure 6–1C), and surrounding the central canal.

In the lower medulla, a dense immunofluorescence occurs in the substantia gelatinosa of spinal nerve V and the nucleus commissuralis; opiate receptor distribution shows a similar pattern (Figure 6–3). A network of fibers also occurs in the reticular formation (Figure 6–3), especially laterally.

At a more anterior portion of the medulla, corresponding to the level of the area postrema, immunofluorescent terminals and fibers are highly concentrated in the nucleus of the solitary tract, in the nucleus originis dorsalis of the vagus, and in the nucleus of nerve XII (Figure 6–3). The nucleus ambiguus has a fairly dense reticular network of fibers and terminals (Figure 6–2D). Less dense fibers occur ventrally to the hypoglossal nucleus, while a

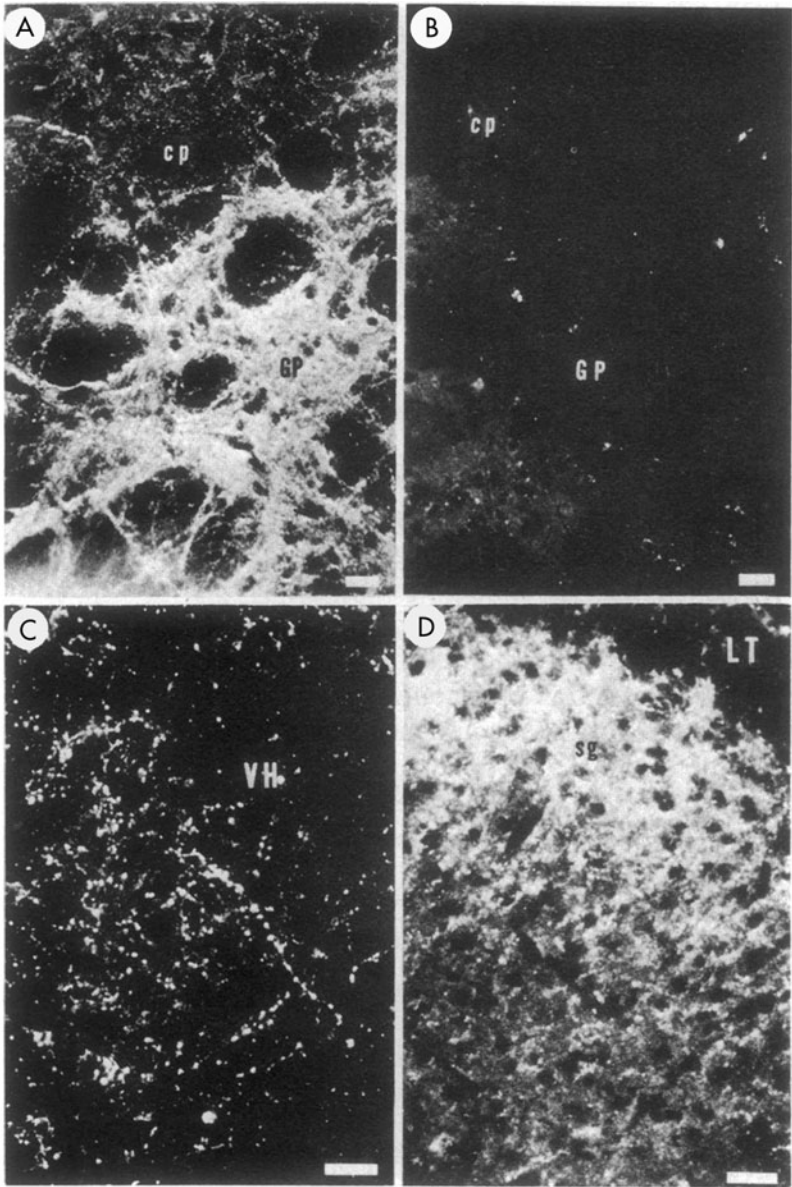


FIGURE 6-1. Immunofluorescence micrographs of (A and B) the globus pallidus (GP) and nucleus caudatus-putamen (cp) and of the cervical spinal cord showing (C) the ventral horn (VH) and (D) Lissauer's Tract (LT) and substantia gelatinosa (sg).

Micrographs (A) and (B) were taken from serial sections, but the primary serum used for staining in (B) was previously adsorbed overnight with 1 mM Leu-enk to establish a control. All sections were stained with rabbit antiserum against Leu-enk. Bars=25 μ m.

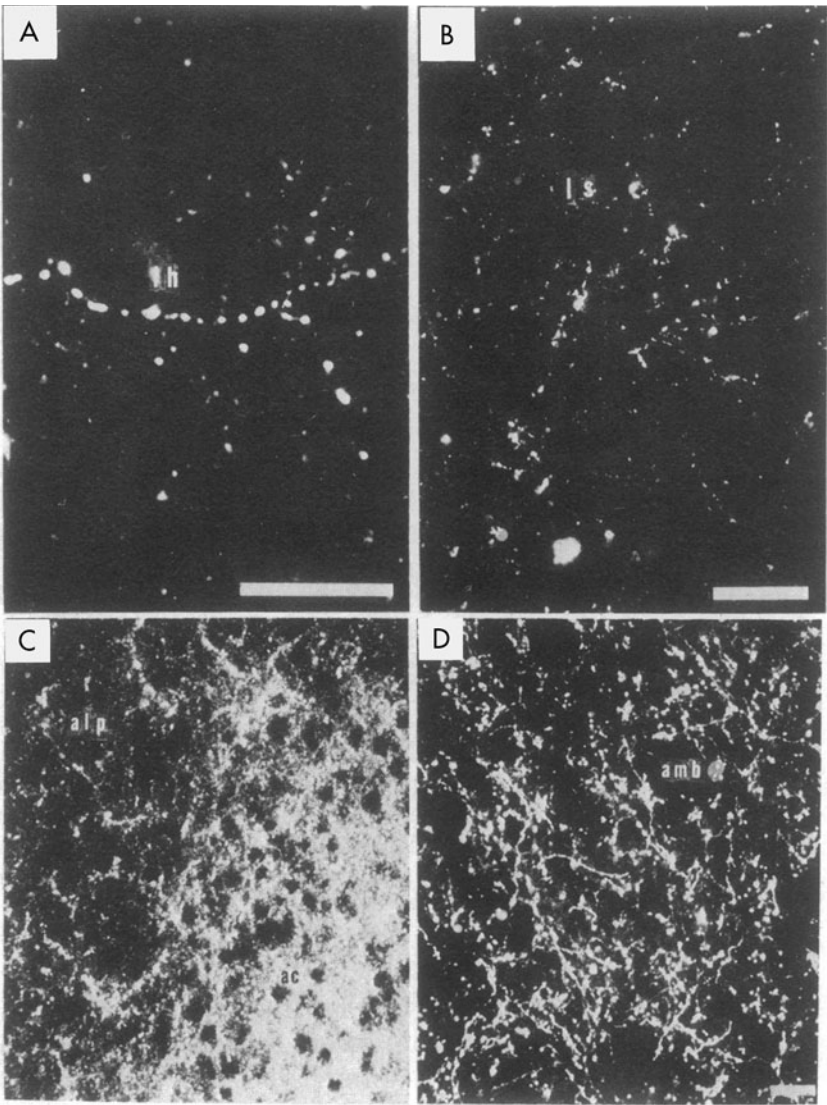


FIGURE 6-2. Immunofluorescence micrographs of (A) the lateral hypothalamus (lh), (B) the lateral septum (ls), (C) the central (ac) and lateral posterior (alp) amygdaloid nuclei, and (D) nuclei ambiguus (amb).

All sections were stained with rabbit antiserum against Leu-enk except for (C), where guinea pig antiserum was used. Bars=25 μ m.

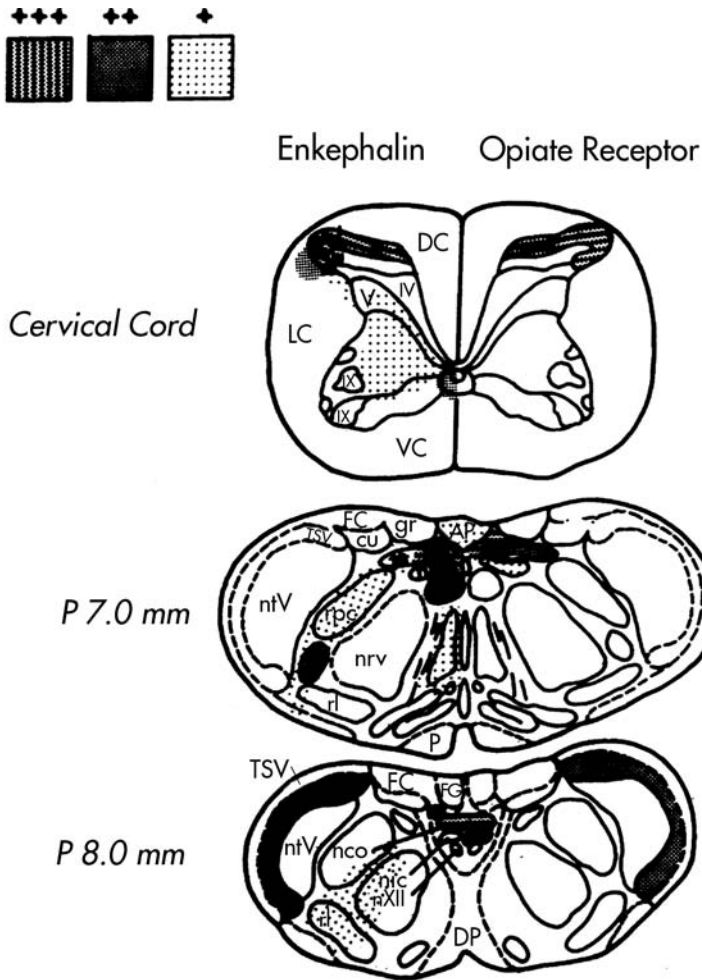


FIGURE 6-3. Distribution of the opiate receptor and enkephalin in the cervical cord and lower medulla.

Abbreviations: amb, nucleus ambiguus; AP, area postrema; cu, nucleus cuneatus; DC, dorsal column; DP, decussatio pyramidis; FC, fasciculus cuneatus; FG, fasciculus gracilis; gr, nucleus gracilis; io, nucleus olivaris inferior; LC, lateral column; nco, nucleus commissuralis; nic, nucleus intercalatus; nrv, nucleus reticularis medullae oblongatae pars ventralis; nts, nucleus tractus solitarius; ntV, nucleus tractus spinalis nervi trigemini; nX, nucleus originis dorsalis vagi; nXII, nucleus originis nervi hypoglossi; P, tractus corticospinalis; rl, nucleus reticularis lateralis; rpc, nucleus reticularis parvocellularis; sgV, substantia gelatinosa trigemini; ts, tractus solitarius; TSV, tractus spinalis nervi trigemini; VC, ventral column. Drawings and coordinates of lower medullary regions are after ref 27. Cervical cord drawing is from ref. 28, and laminae are according to ref. 29. Opiate receptor densities are from ref. 9.

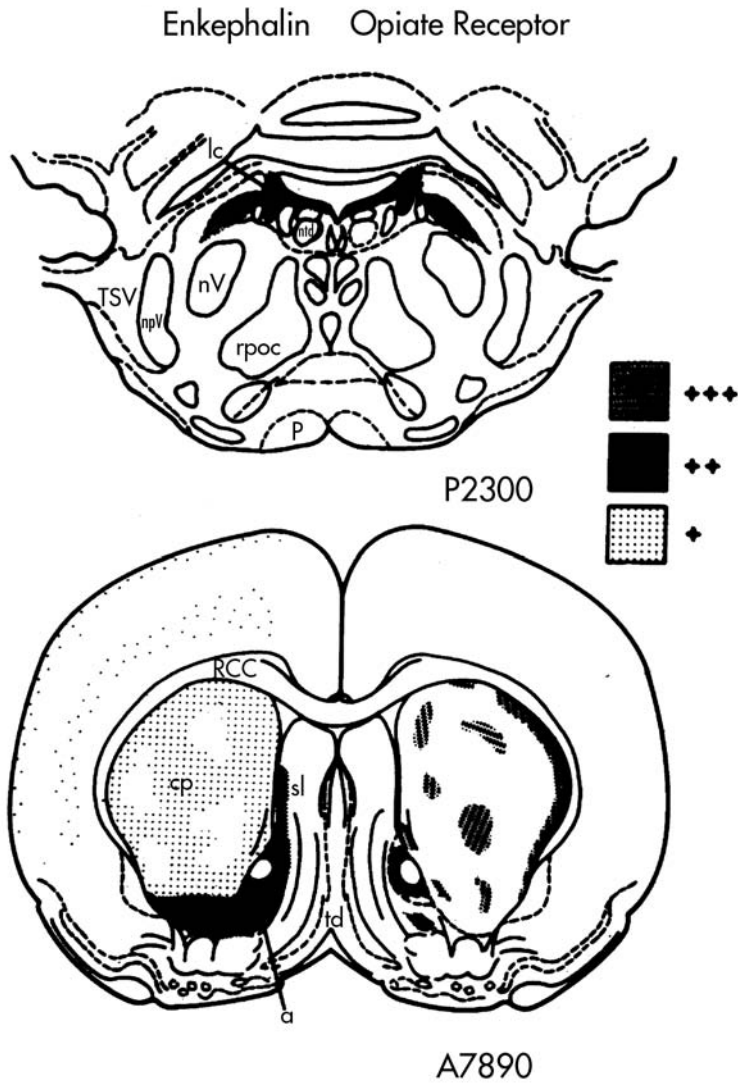


FIGURE 6-4. Distribution of opiate receptors and enkephalin.

Stereotaxic levels are after ref. 30. Opiate receptor distribution is from refs. 8 and 31. Abbreviations: a, nucleus accumbens; cp, nucleus caudatus-putamen; lc, locus coeruleus; npV, nucleus principalis nervi trigemini; ntd, nucleus tegmenti dorsalis Guden; nV, nucleus originis nervi trigemini; P, tractus corticospinalis; RCC, radiatio corporis callosi; rpoc, nucleus reticularis pontis caudalis; sl, nucleus septi lateralis; td, nucleus tractus diagonalis (Broca); TSV, tractus spinalis nervi trigemini.

higher density system passes laterally from the nucleus of the solitary tract (Figure 6-3).

In the brain stem at the level of the locus coeruleus, immunofluorescent terminals and fibers are most highly concentrated in the floor of the fourth ventricle, with a somewhat lesser density within and surrounding the locus coeruleus itself (Figure 6-4). Just anterior to the locus coeruleus, immunofluorescence is most highly localized to the parabrachial nuclei and the floor of the fourth ventricle (Figure 6-5).

In the lower midbrain, fluorescence is most pronounced in the gray matter surrounding the cerebral aqueduct with some lateral extensions into the tegmentum of the midbrain. Moderate fluorescence is also noted in a narrow band near the dorsal portion of the inferior colliculi and overlying the dorsal and median raphe nuclei (Figure 6-5).

Within the thalamus, immunofluorescence is highest in medial dorsal areas, especially those surrounding the ventricles, while lateral thalamic regions are almost devoid of fluorescence. The midline nuclei of the thalamus have intense immunofluorescence, especially the periventricular nucleus *retundocellularis*. At more anterior levels of the thalamus, fluorescence is high in the midline structures, notably the nucleus *parataenialis* and the medullary laminae of the thalamus, especially the internal laminae (Figure 6-5). In contrast to the dense fluorescence of the dorsal and medial areas of the thalamus, most ventral and lateral regions exhibit sparse fluorescence. The *zona incerta* displays substantial immunofluorescence (Figure 6-5), while a network of fluorescent terminals covers the lateral edge of the medial habenula.

Several hypothalamic structures, especially periventricular and ventral portions, are rich in enkephalin immunofluorescence, while certain nuclei fail to display any fluorescence. The periventricular nucleus and a dense band along the ventral floor of the hypothalamus are highly fluorescent, while the supraoptic nucleus, mammillary bodies, arcuate nucleus, and suprachiasmatic nuclei display much sparser immunofluorescence. The only white matter of the hypothalamus displaying substantial fluorescence is the infundibulum; the fornix, optic tract, and optic chiasm lack immunofluorescence (Figure 6-5).

Immunofluorescence in the amygdala is uneven. At all levels, the central nucleus of the amygdala displays dense immunofluorescence while the corticomedial and basolateral areas of the amygdala have lower levels (Figures 6-2C and 6-5). Moderate to low fluorescence occurs lateral to the central amygdala in an area just ventral to the caudate-putamen, possibly in continuity with the immunofluorescent fibers of the ventral caudate-putamen.

Considerable variations in immunofluorescence occur within the basal ganglia. The globus pallidus is the most intensely fluorescent structure

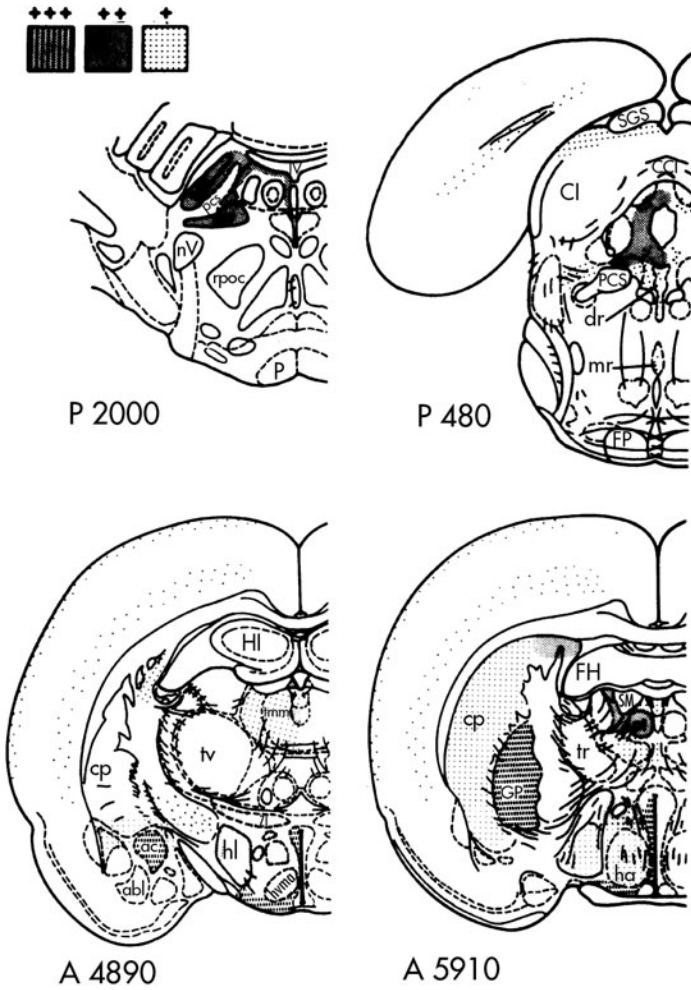


FIGURE 6-5. Distribution of enkephalin.

Stereotaxic levels are from ref. 30. Abbreviations: abl, nucleus amygdaloideus basalis, pars lateralis; ac, nucleus amygdaloideus centralis; cp, nucleus caudatus-putamen; dr, nucleus dorsalis raphes; ha, nucleus anterior (hypothalami); hl, nucleus lateralis (hypothalami); hma, nucleus ventromedialis (hypothalami), pars anterior; mr, nucleus medianus raphes; npd, nucleus parabrachialis dorsalis; npv, nucleus parabrachialis ventralis; nV, nucleus originis nervi trigemini; pt, nucleus parataenialis; rpoc, nucleus reticularis pontis caudalis; tmm, nucleus medialis thalami, pars medialis; tr, nucleus reticularis thalami; tv, nucleus ventralis thalami; CCI, commissura colliculorum inferiorum; CI, colliculus inferior; FH, fimbria hippocampi; FP, fibrae pyramidales; GP, globus pallidus; HI, hippocampus; IV, nervus trochlearis; P, tractus corticospinalis; PCS, pedunculus cerebellaris superior; SGS, stratum griseum superficiale colliculi superioris; SM, stria medullaris thalami; ZI, zona incerta.

observed in the brain (Figures 6–1 and 6–5). Within the globus pallidus, fluorescent fibers and terminals occur in a dense, reticular network surrounding the penetrating nonfluorescent fiber bundles (Figure 1A). Within the caudate-putamen, a somewhat patchy immunofluorescence is largely confined to dorsal and ventral areas with relatively sparse fluorescence in the remainder of the caudate-putamen (Figures 6–4 and 6–5). The nucleus accumbens and the interstitial nucleus of the stria terminalis show moderately dense networks of fibers, which are densest in regions adjacent to the anterior commissure. Densely fluorescent fibers and terminals are observed within the lateral septum (Figures 6–2B and 6–4). A collection of fluorescent fibers appears to proceed ventrally from the lateral septum, possibly along the striohypothalamic tract. A limited amount of fluorescence is observed in the cerebral cortex, most marked in deeper layers, while hippocampal and cerebellar fluorescence is extremely sparse.

Discussion

In many regions of the central nervous system, enkephalin immunofluorescence and autoradiographic opiate receptor grains are intimately associated. Examples include the dorsal laminae of the spinal cord and brain stem, the nucleus of the solitary tract, nucleus commissuralis, parabrachial nuclei, habenula, and globus pallidus (Figures 6–3 and 6–4). There are discrepancies in the correlation between opiate receptor and enkephalin localizations. (a) Though the caudate-putamen displays high levels of opiate receptor binding in biochemical (14, 15) and autoradiographic (9) experiments as well as substantial endogenous levels of enkephalin in both radioreceptor assays (5, 6) and radioimmunoassay (12), immunofluorescence is not dense in the caudate-putamen. (b) Opiate receptor grains occur in patches throughout the substance of the caudate-putamen, but immunoreactive enkephalin tends to be localized mainly to dorsal and ventral regions. (c) The cerebral cortex contains substantial levels of opiate receptor binding (14, 15), demonstrates specific electrophysiological responses to opiates and enkephalin (16, 17, 18), and possesses moderate levels of enkephalin by radio-receptor and radioimmunoassay (5, 6, 12). However, we have detected relatively sparse enkephalin fluorescence within the cerebral cortex. (d) In the spinal cord, the ventral gray matter and an area surrounding the central canal display fluorescent enkephalin fibers but no detectable opiate receptor autoradiographic grains. (e) In the amygdala, immunofluorescence is most highly concentrated in the central nucleus, whereas autoradiographic receptor grains are more evenly distributed throughout the amygdala.

These discrepancies between densities of enkephalin-like immunofluorescence and of opiate receptors may be rationalized in several ways. Brain

areas where receptor density is disproportionately higher than fluorescence density might contain concentrations of "presynaptic" opiate receptors. These receptors could be localized to axons of neurons whose cells or dendrites receive enkephalin input (9, 19, 20). Alternatively, our technique may fail to visualize enkephalin within certain axons. Brain areas where fluorescence is higher than receptor density could conceivably contain concentrations of axons of "enkephalinergic" neurons that pass through these areas and synapse on opiate receptors elsewhere.

Similar to the distribution of opiate receptors (8, 9, 10), the distribution of immunoreactive enkephalin corresponds to regions that mediate functions that are influenced by opiates. Enkephalin in laminae I and II of the spinal cord and the intralaminar nuclei of the thalamus may relate to integration of pain perception. Enkephalin within vagal nuclei of the medulla might play a role in visceral reflexes affected by opiates, such as the cough and vomiting reflexes. Within the periaqueductal gray, enkephalin may mediate electrical stimulation-induced analgesia (21, 22, 23). Enkephalin in the infundibulum and periventricular nuclei of the hypothalamus may reflect loci where opiates influence endocrine functions. Within certain limbic and cortical areas, especially the central nucleus of the amygdala, enkephalin might be associated with the euphoric effects of opiates.

The distribution of enkephalin immunofluorescence that we have observed is similar to that noted by Elde et al. (11). Though we have detected immunofluorescence in all regions where Elde et al. (11) reported fluorescence, we have also observed networks of fibers and terminals not reported by Elde et al. (11), such as the locus coeruleus and the floor of the fourth ventricle, where we observed dense staining.

The enkephalin antisera we have used display little cross-reactivity with larger opioid peptides, α -endorphin and β -endorphin (12). Since radioimmunoassay has shown that rat brain levels of α -endorphin and β -endorphin are lower than those of the enkephalins (J. Rossier, R. Guillemin, and F. Bloom, personal communication), it is unlikely that the immunofluorescence observed in the present study is attributable to these endorphins. These larger opioid peptides occur in very high concentrations in the pituitary (24, 25, 26).

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Part III

DOPAMINE RECEPTORS AND INFLUENCES OF NEUROLEPTICS

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COMMENTARY

Dopamine Receptor Binding and Its Therapeutics

George Aghajanian

Radiolabeled receptor ligand binding has become so routine it is difficult to fully appreciate the excitement created in the mid-1970s by this series of groundbreaking studies directly linking dopamine receptors to antipsychotic drug action. It should be noted that these studies were done only a short time after the Snyder laboratory had pioneered the quantification of opioid receptors by the radiolabeled ligand method.

The first of this series of dopamine receptor papers, published by Creese, Burt, and Snyder in 1976 (1; Chapter 7), was prompted by the realization that there were serious discrepancies challenging the prevailing view that neuroleptic drugs produced their antipsychotic effect by blocking dopamine receptors. Animal behavioral studies had shown that both phenothiazines (e.g., chlorpromazine) and butyrophenones (e.g., haloperidol), the main types of neuroleptics known at the time, blocked dopamine-mediated behaviors in proportion to their clinical potency. However, published biochemical studies did not readily fit the model. In particular, phenothiazines had been found to be more potent relative to butyrophenones in blocking a dopamine-sensitive adenylyl cyclase than would be expected by behavioral or clinical potency. The discovery in the Snyder laboratory of saturable, specific dopamine receptor binding in brain membranes suddenly provided a new tool for resolving these discrepancies. Using [^3H]haloperidol as an antagonist ligand for dopamine receptors, Snyder's team found that the rank

order of antipsychotic drug affinity for the [^3H]haloperidol site (K_i s) correlated almost perfectly with clinical potency in psychiatric patients. This was an impressive demonstration of the power of the neurotransmitter receptor binding approach for establishing links between basic receptor pharmacology and behavioral as well as clinical phenomena.

Over the years, I have had occasion to look back more than once at the original reprint of the paper by Creese et al., now yellowed and still in my files. My return visits were not prompted so much by the central message, which was quite straightforward. Rather, it was certain incidental features that did not fit so well. It was puzzling, for example, that there was such a poor correlation with clinical potency when [^3H]dopamine rather than [^3H]haloperidol was used as the ligand. At the time, this result was attributed to a difference in agonist versus antagonist properties of the dopamine binding sites. Another conundrum was that the binding data still did not explain why the otherwise potent butyrophenones such as haloperidol were such weak inhibitors of dopamine-activated adenylyl cyclase.

However, by the time the third paper in this series appeared in 1981 (2; Chapter 9), the real reasons for this apparent anomaly were becoming understood: work from a number of laboratories revealed that there were at least two different dopamine receptors: one that was coupled to adenylyl cyclase (termed D_1) and another, presumably not linked to adenylyl cyclase, that was labeled by butyrophenones such as [^3H]haloperidol (termed D_2). Since [^3H]dopamine would bind to both receptors, this would confound any rank-order-based affinity for just one of the dopamine receptor subtypes. This differentiation was confirmed years later by the emergence of receptor cloning when it became clear that D_1 and D_2 represented two different families of dopamine receptors, D_1 , D_5 , and D_{off} being in a G_s -coupled group and D_2 , D_3 , and D_4 in a G_i/G_o G protein-coupled group.

But even at the time of Sol's 1981 review, it was realized that there were important therapeutic implications arising from the identification of two distinct types of dopamine receptor. Sol concluded: "The fact that butyrophenones are quite potent in...causing extrapyramidal side effects, and in relieving schizophrenic symptoms indicates that all of these actions involve DA-2 receptors. Indeed, the DA-1 sites appear to be receptors in search of a therapeutic function" (2).

Sol's conclusion that D_1 antagonism did not contribute to therapeutic effects of antipsychotic drugs was overlooked by some investigators who took a different tack, and a decade later, in the early 1990s, a highly selective D_1 antagonist had been developed by industry and tested in a number of clinical trials for antipsychotic activity. Uniformly, however, the results showed that the D_1 antagonist was not only lacking in therapeutic efficacy, it actually could worsen the symptoms of schizophrenia. In retrospect, this should not

have been such a surprising result given the preclinical studies in primates in the laboratory of the late Pat Goldman-Rakic showing that an optimal level of D_1 receptor stimulation in prefrontal cortex is important for cognitive function and that blockade of D_1 receptors in prefrontal cortex can be detrimental. At the present time, things have come full circle as D_1 agonists rather than antagonists have been proposed by several groups as potential therapeutic agents in schizophrenia, especially aimed at overcoming negative symptoms such as poor socialization and cognitive impairments. Thus, one can look back at the 1981 Snyder review as setting an agenda that extends to the present time for differentiating the function of D_1 and D_2 receptors.

It was also noted by Creese et al. (1) that two drugs on the list, thioridazine and clozapine, were much less likely to induce extrapyramidal side effects than standard antipsychotics. It had been proposed previously by others that this might be explained by a differential action on dopamine receptors in the corpus striatum versus other parts of the brain that may be more closely linked to their antipsychotic action. However, a regional comparison of competition for [3 H]haloperidol binding showed no such differences. Another possibility that Creese et al. mention was that the anticholinergic properties of these drugs might account for their low incidence of extrapyramidal side effects. Nevertheless, at the time, these drugs were still regarded as otherwise typical in that their "relative affinities...for [3 H]haloperidol binding correspond reasonably well with their clinical potencies." The key phrase here is *reasonably well*, which suggests that the authors may have had some reservations about how good the fit was. Of particular note is the fact that clozapine's K_i for [3 H]haloperidol binding was 10 times greater than that of chlorpromazine, yet the average clinical daily dose is only twice that of chlorpromazine (see Table 7-1 in this volume). It would appear that the clinical potency of clozapine was greater than one would expect from its K_i for [3 H]haloperidol binding. At the time, such isolated deviations did not attract much attention, but in later years an accumulation of clinical data showed that clozapine might be different from typical antipsychotic drugs with respect to efficacy, particularly in its ability to ameliorate negative symptoms of schizophrenia. This, together with clozapine's low (perhaps zero) risk for producing extrapyramidal side effects and tardive dyskinesia, eventually led to the concept that it was an "atypical" antipsychotic drug.

So what might be responsible for clozapine's atypical properties? Subsequent receptor binding studies from the Snyder laboratory and other labs have shown that clozapine interacts with numerous non- D_2 receptors. These include D_1 receptors (possibly as an agonist), at least three subtypes of serotonin receptors (as antagonists at 5-HT_{2A} and 5-HT_{2C} and as a partial agonist at 5-HT_{1A} receptors), muscarinic cholinergic receptors (as antagonist or ag-

onist, depending on subtype), H_1 histamine receptors, and two types of adrenergic receptor (α_1 and α_2). Even today, it is not well understood which receptor(s) may be responsible for clozapine's atypicality. Nevertheless, the mere existence of this rich multiplicity of receptor interactions spawned an effort in industry to develop new atypical antipsychotic drugs that could mimic clozapine in having activity as antagonists (or agonists) at one or more non- D_2 receptors. This effort was partly driven by the need to have drugs that avoided certain of clozapine's side effects such as sedation and especially its tendency to produce agranulocytosis. Eventually, these efforts led to the present-day situation where the most frequently prescribed antipsychotic drugs are of the atypical variety. But it should be remembered that these developments are rooted in Creese et al. (1), the very first paper showing a relationship between clinical potency and dopamine receptor binding.

The remaining paper in the series, published by Burt, Creese, and Snyder in 1977 (3; Chapter 8), addresses a different issue, the development of dopamine receptor supersensitivity after prolonged treatment with antipsychotic drugs. It had been speculated that such supersensitivity may underlie tardive dyskinesia, a motor disturbance that can occur in patients following long-term treatment with antipsychotic drugs. Animal behavioral studies had shown that supersensitivity occurs following termination of antipsychotic drug administration or by depleting brain levels of dopamine with the drug reserpine. This effect was manifested by increased motor responses to dopamine agonist drugs such as apomorphine. Lesions of the dopaminergic nigrostriatal pathway also resulted in such enhanced responses, indicating it was a phenomenon akin to classical denervation supersensitivity. However, the mechanism of this enhanced responsivity was unclear since, in addition to direct changes at a dopamine receptor level, changes in other neuronal pathways could have been responsible for the enhanced responses. Obviously, this presented itself as another excellent opportunity for applying the dopamine ligand binding procedure to distinguish between these biologically distinct alternative hypotheses. Using [3H]haloperidol as the ligand, an approximately 25% elevation of dopamine receptor binding in the striatum was seen five days after cessation of chronic haloperidol treatment. This was shown to be due to an upregulation in the number of dopamine receptors without any increase in affinity. It was also shown that the upregulation of dopamine receptors was persistent and did not return to normal level until about 2.5 weeks after termination of treatment. These results suggest that a prolonged upregulation of dopamine receptors after long-term treatment with antipsychotic drugs could underlie the development of tardive dyskinesia in schizophrenic patients. A more difficult question has been whether such an upregulation occurs in drug-naïve schizophrenic patients, possibly contributing to the pathogenesis of the illness itself. However, after numer-

ous studies over many years involving in a large number of laboratories, a consensus on this issue has not been achieved.

Apart from these specific issues—some resolved, some not—it can be said that in the three decades since their publication in 1976–1981, no aspect of the dopamine receptor and antipsychotic drug field has been untouched by these three landmark papers from the Snyder laboratory. This is the best testament to their lasting value.

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CHAPTER 7

Dopamine Receptor Binding Predicts Clinical and Pharmacological Potencies of Antischizophrenic Drugs

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Neuroleptic phenothiazine and butyrophenone drugs are thought to alleviate schizophrenic symptoms and induce parkinsonism-like extrapyramidal side effects by blocking dopamine receptors in the brain (1). While molecular modeling indicates how phenothiazines can assume the preferred conformation of dopamine, the conformation of butyrophenones at their receptor sites is unclear (2). Nevertheless, in behavioral tests both phenothiazines and butyrophenones block dopamine-mediated behaviors induced by amphetamine and apomorphine in proportion to their clinical potency (3).

A dopamine-sensitive adenylate cyclase, localized to areas of the brain rich in dopamine terminals, appears to be associated with the postsynaptic dopamine receptor and might therefore predict potencies of dopamine antagonists (4). While there are some correlations between the pharmacological potencies of phenothiazines and their inhibition of the dopamine-sensitive

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adenylate cyclase, major discrepancies exist for butyrophenones (5). Although butyrophenone neuroleptics such as spiroperidol and pimozide are weaker than chlorpromazine in inhibiting the dopamine-sensitive cyclase, *in vivo* behavioral and clinical data show them to be considerably more potent than chlorpromazine (3). These discrepancies can be construed as challenges to hypotheses that antischizophrenic drugs produce their therapeutic effects by blocking postsynaptic dopamine receptors; indeed it has been suggested instead that the drugs might act by inhibiting dopamine release (6).

Recently, dopamine receptor binding has been demonstrated in brain membranes by labeling the receptor both with the agonist [^3H]dopamine and with the antagonist [^3H]haloperidol (7). The regional distribution of receptor binding and the relative potencies of catecholamines and a variety of drugs ensure that binding of these ligands is associated with postsynaptic dopamine receptors, while the failure of destruction of dopamine nerve terminals to decrease binding indicates that presynaptic receptors are not involved. [^3H]Dopamine and [^3H]haloperidol appear to label distinct agonist and antagonist states of the receptor, respectively (8). Thus dopamine and other agonists have a much greater affinity for dopamine than haloperidol sites, while the reverse holds true for dopamine antagonists. We now report that the relative affinities of an extensive series of butyrophenones, phenothiazines, and other dopamine antagonists in competing for [^3H]haloperidol binding to the dopamine receptor predict the pharmacological activities of these drugs in animal behavioral tests and their clinical potencies in psychiatric patients.

Methods were essentially as described previously (7). Homogenates (Brinkmann Polytron) of fresh calf caudate in cold tris(hydroxymethyl)aminomethane (Tris) buffer were washed twice by centrifugation. The final resuspension (in cold 50 mM Tris buffer containing 0.1% ascorbic acid, 10 μM pargyline, and ions as follows: 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , giving a final pH of 7.1 at 37°C) was warmed to 37°C for 5 minutes and returned to ice. Each tube received 2 mL of caudate suspension (20 mg, wet weight) and contained 5 nM [^3H]dopamine ([ethyl-1- ^3H]dopamine, 8.4 c/mmole, New England Nuclear) or 2 nM [^3H]haloperidol (0.86 c/mmole, custom tritiated by New England Nuclear and purified by a thin-layer chromatography) and various concentrations of nonradioactive drugs. Triplicate tubes were incubated at 37°C for 10 minutes and rapidly filtered under vacuum through Whatman GF/B filters with two 5-mL rinses of cold buffer. The filters were counted by liquid scintillation spectrometry.

Saturable or specific binding of [^3H]dopamine was measured as the excess over blanks taken in the presence of 1 μM dopamine or 10 μM (+)-butaclamol. Blank tubes for [^3H]haloperidol binding contained 100 μM

dopamine or 0.1 μM (+)-butaclamol. By these criteria half of the total [^3H]dopamine binding and about 40% of the [^3H]haloperidol binding were specific. Binding of [^3H]dopamine has a dissociation constant, K_D , of about 20 nM, while that of [^3H]haloperidol binding is about 2 nM.

In general, butyrophenones and related drugs are the most potent class of neuroleptics in treating schizophrenia and in antagonizing dopamine-mediated behaviors in animals. The most potent of the drugs examined in this study, spiroperidol, substantially protects dogs against apomorphine-induced emesis and rats against amphetamine-induced stereotyped behavior at doses under 0.05 $\mu\text{mole/kg}$, a dose level similar to that used therapeutically in humans (Table 7-1). Spiroperidol is also the most potent inhibitor of [^3H]haloperidol binding with a value of the inhibition constant, K_i , indicating 50% receptor occupation, of 0.25 nM. It thus possesses a 5-fold higher affinity for [^3H]haloperidol binding than fluphenazine, a potent phenothiazine, a 40-fold greater affinity than chlorpromazine, and a 125-fold to 950-fold greater affinity than the weak neuroleptics pipamperone, promazine, and promethazine (Table 7-1). There is an excellent correlation between the molar pharmacological potencies of the butyrophenones, phenothiazines, and related compounds in animals and man and their affinities for [^3H]haloperidol binding sites. On a log-log graph (not shown) of the data in Table 7-1, the affinities of these 25 drugs for [^3H]haloperidol binding sites display a correlation coefficient (r) of 94 ($P < 0.001$) with pharmacological potencies in antagonizing apomorphine stereotypy and an r of 0.92 ($P < 0.001$) in antagonizing amphetamine stereotypy in rats; an r of 0.93 ($P < 0.001$) with potencies in antagonizing apomorphine-induced emesis in dogs; and an r of 0.87 ($P < 0.001$) with clinical potencies in man. These impressive correlations indicate that affinity for [^3H]haloperidol binding to dopamine receptors is a powerful predictor of clinical activity. The correlations are all the more impressive because binding studies were conducted *in vitro* and animal behavioral and human studies conducted *in vivo*.

Dopamine receptor blockade in the corpus striatum is presumed to be responsible for extrapyramidal parkinsonism-like side effects of neuroleptic drugs, while antischizophrenic actions may involve dopamine receptors in other brain regions as well (1). Some neuroleptics, such as thioridazine and clozapine, elicit a much lower incidence of extrapyramidal effects than most agents, which could arise from differential influences on dopamine receptors in various areas. This is unlikely, because our regional studies indicate that clozapine, thioridazine, other neuroleptics, and dopamine have about the same affinity for [^3H]haloperidol binding sites in the corpus striatum as in two mesolimbic areas of the brain, the olfactory tubercle and nucleus accumbens (Table 7-2). Neuroleptics also affect the dopamine-sensitive adenylate cyclase to a similar extent in these three areas (4). The relative affinities of

TABLE 7-1. Antischizophrenic drugs: comparison of affinities for [³H]haloperidol and [³H]dopamine binding sites with *in vivo* pharmacological potencies

| Drug | Inhibition of [³ H]haloperidol binding, K_i (nM) | Inhibition of apomorphine stereotypy in rat, ID_{50} (μmole/kg) | Inhibition of apomorphine-induced emesis in dog, PD_{50} (μmole/kg) | Inhibition of amphetamine stereotypy in rat, ID_{50} (μmole/kg) | Average clinical daily dose (μmole/kg) | Inhibition of [³ H]dopamine binding, K_i (nM) | Rank order, inhibition of [³ H]dopamine binding |
|-----------------|--|---|---|---|--|---|---|
| Spiroperidol | 0.25±0.02 (4) | 0.177 | 0.0006 | 0.051 | 0.058 | 1,400±190 (3) | 16 |
| Benperidol | 0.33±0.02 (4) | 0.118 | 0.0012 | 0.071 | 0.060 | 4,100±540 (4) | 21 |
| Clofluperol | 0.50±0.03 (4) | 0.198 | | 0.117 | 0.077 | 360±20 (3) | 4 |
| (+)-Butaclamol | 0.55±0.09 (8) | | 0.095 | | 2.14 | 70±10 (10) | 1 |
| Fluspirilene | 0.60±0.13 (4) | | | | 0.066 | 1,400±220 (4) | 15 |
| Pimozide | 0.80±0.07 (4) | 0.370 | 0.024 | 0.242 | 0.108 | 4,100±1,140 (4) | 22 |
| Trifluoperidol | 0.95±0.19 (3) | 0.067 | 0.016 | 0.056 | 0.096 | 740±20 (3) | 10 |
| Droperidol | 1.0±0.10 (4) | 0.185 | 0.003 | 0.095 | | 880±80 (3) | 12 |
| α-Flupenthixol | 1.1±0.22 (4) | 0.867 | | 0.650 | 0.099 | 180±30 (8) | 3 |
| Fluphenazine | 1.2±0.12 (6) | 0.255 | 0.012 | 0.196 | 0.168 | 180±30 (5) | 2 |
| Bromperidol | 1.4±0.15 (4) | 0.324 | 0.038 | 0.126 | 0.153 | 600±90 (3) | 7 |
| cis-Thiothixene | 1.4±0.11 (4) | 1.42 | | 0.803 | 0.393 | 540±140 (6) | 6 |
| Haloperidol | 1.5±0.14 (9) | 0.532 | 0.050 | 0.101 | 0.152 | 650±90 (4) | 8 |
| Moperone | 1.9±0.26 (4) | 0.638 | 0.050 | 0.059 | 0.802 | 1,200±160 (4) | 14 |
| Triflupromazine | 2.1±0.12 (4) | 4.62 | 0.50 | 0.746 | 4.59 | 530±80 (5) | 5 |
| Trifluoperazine | 2.1±0.34 (4) | 1.14 | 0.08 | 0.520 | 0.297 | 740±80 (5) | 9 |
| Fluanisone | 3.8±0.80 (4) | 6.17 | 0.40 | 0.757 | 3.44 | 800±180 (4) | 11 |

TABLE 7-1. Antischizophrenic drugs: comparison of affinities for [³H]haloperidol and [³H]dopamine binding sites with *in vivo* pharmacological potencies (*continued*)

| Drug | Inhibition of [³ H]haloperidol binding, K_i (nM) | Inhibition of apomorphine stereotypy in rat, ID ₅₀ (μmole/kg) | Inhibition of apomorphine-induced emesis in dog, PD ₅₀ (μmole/kg) | Inhibition of amphetamine stereotypy in rat, ID ₅₀ (μmole/kg) | Average clinical daily dose (μmole/kg) | Inhibition of [³ H]dopamine binding, K_i (nM) | Rank order, inhibition of [³ H]dopamine binding |
|---|--|--|--|--|--|---|---|
| Penfluridol | 5.6±1.40 (7) | | | | 0.466 | 1,600±310 (4) | 17 |
| Azaperone | 10.0±0.6 (4) | 27.4 | | 9.16 | | 1,700±290 (4) | 18 |
| Chlorpromazine | 10.3±0.2 (5) | 18.3 | 2.0 | 3.09 | 12.0 | 900±200 (7) | 13 |
| Thioridazine | 14.0±0.2 (5) | | | | 12.6 | 1,780±332 (4) | 19 |
| Pipamperone | 31.3±5.2 (4) | 635 | 3.5 | 11.1 | 11.1 | 4,900±500 (4) | 23 |
| Promazine | 71.5±3.2 (4) | >250 | 60 | 99.6 | 33.3 | 7,100±1,640 (8) | 24 |
| Clozapine | 100±6 (6) | | | | 24.6 | 1,890±340 (5) | 20 |
| Promethazine | 238±32 (4) | | | | | 12,000±3,600 (7) | 25 |
| Correlation with [³ H]haloperidol binding | | $r=0.94$ $P<0.001$ | $r=0.93$ $P<0.001$ | $r=0.92$ $P<0.001$ | $r=0.87$ $P<0.001$ | $r=0.58$ $P<0.01$ | |
| Correlation with [³ H]dopamine binding | | $r=0.46$ $P<0.05$ | $r=0.22$ $P>0.05$ | $r=0.41$ $P>0.05$ | $r=0.27$ $P>0.05$ | | |

Note. Drugs are listed in order of affinity for [³H]haloperidol binding sites of calf striatal membranes. For each drug competition of binding of both ligands was measured at two to four concentrations of drug, and 50% inhibitory concentrations, IC₅₀, were derived by log-probit analysis. These values were converted to apparent K_i 's according to the equation $K_i = IC_{50}/(1 + C/K_D)$, where C is the concentration of radioactive ligand and K_D is its dissociation constant. Each value is the mean±standard error of the mean for three to ten determinations (N is given in parentheses). *In vivo* animal data and clinical potencies were calculated from published results (12, 13); ID₅₀ and PD₅₀ are 50% inhibitory dose and 50% protective dose, respectively.

TABLE 7-2. Regional comparison of competition for [³H]haloperidol binding by antischizophrenic drugs

| Drug | K_i (nM) | | |
|--------------|-----------------|--------------------|-------------------|
| | Corpus striatum | Olfactory tubercle | Nucleus accumbens |
| Dopamine | 550±100 (6) | 700±40 (2) | 475±75 (2) |
| Haloperidol | 1.4±0.1 (5) | 0.8±0.3 (3) | 0.8±0.1 (2) |
| Pimozide | 0.6±0.2 (2) | 0.6±0.04 (2) | 0.6±0.1 (2) |
| Fluphenazine | 0.6±0.1 (6) | 0.5±0.05 (2) | 0.8±0.2 (2) |
| Thioridazine | 14±1.3 (5) | 14±2 (3) | 16 (1) |
| Clozapine | 100±6 (6) | 64±24 (2) | 80±0 (2) |

Note. Experiments were performed as described in Table 7-1. Fresh calf brain was dissected into regions and assayed, using the same drug dilutions. Except for clozapine and thioridazine, striatal data are from other experiments than those in Table 7-1. Each value is the mean±standard error of the mean (*N* is given in parentheses).

clozapine and thioridazine for [³H]haloperidol binding correspond reasonably well with their clinical potencies (Table 7-1). The anticholinergic properties of these drugs may well account for their low incidence of extrapyramidal effects (1).

The great potency of butyrophenones and phenothiazines in competing for [³H]haloperidol binding to the dopamine receptor contrasts with their relatively low potency in competing for the binding of [³H]dopamine. The absolute potencies of all drugs correlate significantly for the two types of binding ($r=0.58$, $P<0.01$), although there are discrepancies in rank order for some drugs. Competition of the drugs for [³H]dopamine binding correlates much less well than for [³H]haloperidol binding with the behavioral activities of these agents in rat (for apomorphine antagonism $r=0.46$, $P<0.05$, and for amphetamine antagonism $r=0.41$, $P>0.05$) and dog (for apomorphine antagonism $r=0.22$, $P>0.05$) and with their clinical potencies ($r=0.27$, $P>0.05$).

We have attributed the fact that phenothiazines and butyrophenones are much less potent in competing for [³H]dopamine than for [³H]haloperidol binding to the selective labeling by [³H]dopamine of the agonist state of the dopamine receptor and the labeling by [³H]haloperidol of the antagonist state (7). One might expect that the relative affinities of drugs for [³H]dopamine and [³H]haloperidol sites would indicate the extent to which the drugs are pure agonists, pure antagonists, or mixed agonist-antagonists. This is confirmed by data showing that *D*-lysergic acid diethylamide, a mixed agonist-antagonist of the dopamine-sensitive adenylate cyclase (9), has similar affinities for both [³H]dopamine and [³H]haloperidol binding sites (10).

Conceivably the different relative affinities of antischizophrenic drugs for [^3H]dopamine and [^3H]haloperidol binding sites indicate that these drugs vary in how they affect the dopamine receptor. For instance, some may be more "pure" antagonists than others.

The data reported here demonstrate an extremely close correlation between the clinical and pharmacological potencies of butyrophenones and phenothiazines and their affinities in competing for the binding of [^3H]haloperidol to dopamine postsynaptic receptors. This result argues that these drugs do act by blocking postsynaptic dopamine receptors. Reasons for discrepancies between results with the dopamine-sensitive adenylate cyclase and the *in vivo* and binding data are unclear but may be related to variable degrees of coupling of dopamine receptor sites with the adenylate cyclase (11).

Labeling of postsynaptic dopamine receptors by [^3H]haloperidol provides a simple, sensitive, and specific means for screening phenothiazines, butyrophenones, and related agents as potential antischizophrenic drugs.

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For each drug, the midpoint values of listed ranges of daily dose were averaged and converted to micromoles per kilogram, assuming a human weight of 70 kg.

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CHAPTER 8

Antischizophrenic Drugs

Chronic Treatment Elevates Dopamine Receptor Binding in Brain

David R. Burt

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Chronic treatment with antischizophrenic neuroleptic drugs produces motor abnormalities that appear related to the dopamine neuronal systems in the brain in both man and animals. Tardive dyskinesia, characterized by abnormal movements of facial muscles and extremities, is a major complication of long-term treatment with neuroleptic drugs (1). Lowering the dose or terminating the drugs frequently worsens these symptoms, while increasing the dose may alleviate symptoms. Since a major action of neuroleptics is blockade of dopamine receptors in the brain, speculations have linked tardive dyskinesia with a supersensitivity of dopamine receptors after prolonged blockade by chronic drug administration. This would explain why a reduction of dose worsens symptoms while a dose increase temporarily reverses motor abnormalities. Studies in animal models of tardive dyskinesia support the notion that the increased motor activity reflects supersensitivity of dopamine receptors. Rats or mice treated chronically with neuroleptic

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drugs display an enhanced sensitivity to the motor stimulant effects of apomorphine, a direct dopamine receptor agonist, after the neuroleptic treatment is terminated (2, 3, 4). A similar motor supersensitivity to dopamine receptor stimulants is apparent when dopamine synaptic activity is reduced by inhibiting synthesis of dopamine with α -methyltyrosine (2, 4), depleting dopamine storage with reserpine (2, 5), or making lesions in the nigrostriatal dopamine pathway (5, 6).

Dopamine receptors in the brain can be labeled by direct binding of [^3H]haloperidol (7). Binding sites occur only in brain regions rich in dopamine synapses, and the relative potencies of numerous neuroleptic drugs for the binding sites parallel their behavioral activities in animals and man (8). We now report enhanced dopamine receptor binding of [^3H]haloperidol in the corpus striatum of rats treated chronically with neuroleptic drugs.

Binding assays were performed as described (8). Homogenates (Brinkmann Polytron) of fresh rat corpus striatum (40 mg per side) in cold tris (hydroxymethyl) aminomethane (Tris) buffer were washed twice by centrifugation. The final resuspension (in cold 50 mM Tris buffer containing 0.1% ascorbic acid, 10 μM pargyline, 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , and 1 mM MgCl_2 ; final pH of 7.1 at 37°C) was warmed to 37°C for 5 minutes and returned to 4°C. Each tube received 1 mL of tissue suspension (8 mg of wet tissue) and contained 0.2 to 6 nM [^3H]haloperidol (9.6 c/mmole, Janssen Pharmaceutica). Tubes were incubated at 37°C for 10 minutes, and triplicate 0.3-mL portions were rapidly filtered under vacuum through Whatman GF/B filters with two 5-mL rinses of cold buffer. The filters were counted by liquid scintillation spectrometry. Specific binding of [^3H]haloperidol, measured as the excess over blank tubes containing 100 μM dopamine in addition to the above incubation mixture, represented about half of the total binding.

Treatment of rats for 3 weeks (Table 8-1) with the potent butyrophenone neuroleptic haloperidol produces about a 20% increase in specific [^3H]haloperidol binding ($P < 0.0005$). Fluphenazine, one of the most potent phenothiazine neuroleptics, produces a similar increase in binding after administration for 3 weeks. By contrast, treatment for 3 weeks with a substantially higher dose of the phenothiazine promethazine, which lacks antischizophrenic neuroleptic activity (9), fails to significantly enhance [^3H]haloperidol binding. Administration of reserpine, an antischizophrenic drug that depletes brain dopamine, also elicits about a 20% augmentation of [^3H]haloperidol binding. Treatment with the stimulant amphetamine for 3 weeks fails to change receptor binding significantly. In all of these experiments, dopamine receptor binding was assayed 5 to 7 days after termination of drug treatment. This permitted partial elimination of the drugs from the

TABLE 8-1. Effect of chronic drug treatments on [³H]haloperidol binding in the rat

| Injected drug | Number of treated animals | Difference relative to control (%) | P |
|---------------|---------------------------|------------------------------------|---------|
| Haloperidol | 21 | 19±4 | <0.0005 |
| Reserpine | 10 | 23±7 | <0.005 |
| Fluphenazine | 6 | 27±12 | <0.05 |
| Promethazine | 12 | 3±7 | NS |
| Amphetamine | 5 | -2±4 | NS |

Note. Rats were injected subcutaneously with haloperidol (Haldol, 0.5 mg/kg), reserpine (Serpasil, 0.25 mg/kg), fluphenazine (Prolixin, 0.5 mg/kg), promethazine (Phenergan, 2.5 mg/kg), or *d*-amphetamine sulfate (5 mg/kg) daily for 3 weeks and killed 5 to 7 days later. Freshly removed corpora striata were assayed for binding with three concentrations of [³H]haloperidol (0.2 to 1.4 nM). Results for the three concentrations were averaged for each rat. Data for each treated rat were expressed as the percentage difference in specifically bound radioactivity relative to that in a matched uninjected control rat assayed in parallel. Means and standard errors of the mean are given; probability values were computed by the one-tailed *t*-test; NS, not significant. Control values for [³H]haloperidol binding are as given for Table 8-2.

body, as their continued presence might interfere with dopamine receptor binding. The behavioral supersensitivity to apomorphine is demonstrable for at least a week after neuroleptic treatment of rats is terminated (2).

To evaluate the dose characteristics and time course of the changes in receptor binding, we compared the effects of two doses of haloperidol, 0.5 and 5.0 mg per kilogram of body weight, given daily for 1 or 3 weeks (Table 8-2). We measured [³H]haloperidol binding 5, 12, and 17 days after drug treatment was terminated. The augmentation of binding is as great with the lower as with the higher haloperidol dose. Likewise, the increase in binding after 1 week of haloperidol treatment is similar to that after 3 weeks. Twelve days after haloperidol treatment was terminated, the increased binding is less apparent than at 5 days, while at 17 days no increase is detected.

To determine whether the enhanced receptor binding is attributable to an increased number of binding sites or to a change in affinity, we examined tissue samples from individual rats for binding at five concentrations of [³H]haloperidol ranging from 0.2 to 6.0 nM, and analyzed the data by Scatchard plots drawn by the method of least squares. Five days after termination of haloperidol administration (0.5 mg/kg daily for 21 days), the dissociation constant for [³H]haloperidol binding in the corpus striatum does not differ significantly from that for controls (control: $K_D=0.91\pm0.06$ nM, $N=21$; haloperidol treatment: $K_D=0.99\pm0.09$ nM, $N=21$). By contrast, there

TABLE 8-2. Time course and dose dependence of increase in [³H]haloperidol binding after chronic haloperidol treatment

| Treatment | | | [³ H]Haloperidol concentration | | | |
|--------------------------|--------------------|-------------------------|--|----------|-------------------------|----------|
| | | | 0.4 nM | | 0.8 nM | |
| | | | Increase in binding (%) | <i>P</i> | Increase in binding (%) | <i>P</i> |
| Injection period (weeks) | Daily dose (mg/kg) | Drug-free period (days) | | | | |
| 1 | 0.5 | 5 | 19±5 | <0.01 | 36±11 | <0.025 |
| | 5.0 | | 27±7 | <0.01 | 15±9 | NS |
| 3 | 0.5 | 5 | 25±2 | <0.001 | 24±9 | <0.05 |
| | 5.0 | | 17±6 | <0.025 | 31±7 | <0.005 |
| 3 | 0.5 | 12 | 13±14 | NS | 18±6 | <0.05 |
| 3 | 0.5 | 17 | 0±6 | NS | 8±5 | NS |

Note. Rats were injected daily for 1 or 3 weeks with haloperidol, 0.5 or 5 mg/kg, as in Table 8-1, and were killed 5 to 17 days later. Freshly removed striata were assayed for binding with 0.4 and 0.8 nM [³H]haloperidol. Data are expressed as in Table 8-1. Each result is the mean and standard error of the mean for five pairs of rats, paired in order of assay. Probability values were calculated by the one-tailed *t*-test. [³H]Haloperidol binding values at 0.4 and 0.8 nM in control striata were equivalent to 5 and 8 pmole per gram of tissue, respectively.

is a 20% to 25% increase in the total number of binding sites, corresponding to the similar increase observed in receptor binding at low concentrations of [^3H]haloperidol (control: binding = 27.9 ± 1.4 pmole per gram of tissue; haloperidol treatment: binding = 34.4 ± 1.9 pmole/g; $P < 0.005$, Student's *t*-test on paired data).

Both the clinical motor abnormalities seen after prolonged administration of neuroleptics in man and the enhanced response to apomorphine seen after such treatments in rodents suggest that dopamine receptor sites in the corpus striatum may be supersensitive. However, behavioral supersensitivity to dopamine could be produced by a variety of other mechanisms, such as effects on nondopamine neuronal systems, metabolic changes in cells postsynaptic to dopamine neurons, or behavioral conditioning phenomena (10).

Our data indicate that the motor changes seen after chronic neuroleptic treatment are associated with an increase in the number of dopamine receptors. This increase in the number of [^3H]haloperidol binding sites is consistent with the behavioral supersensitivity to apomorphine in rats treated with neuroleptics on a similar dose schedule. The greater relative enhancement of apomorphine stimulated effects in such rats (2) compared to the increased [^3H]haloperidol binding found here suggests that postreceptor components may also be involved in the increased behavioral response. The activity of a dopamine-sensitive adenylate cyclase in the corpus striatum is not altered in mice treated chronically with neuroleptics (3). Moreover, the ability of apomorphine to elevate striatal adenosine 3', 5'-monophosphate concentrations *in vivo* is unaltered in these mice (3). This apparent discrepancy between the increased receptor binding—reflecting the recognition sites of the dopamine receptor—and adenylate cyclase activity accords with other data indicating that receptor binding sites and associated adenylate cyclase may be distinct entities (8, 11).

Behavioral supersensitivity to apomorphine is more pronounced after lesions of the nigrostriatal dopamine pathway than after chronic treatment with neuroleptic drugs. We have observed enhancement of dopamine receptor binding in the corpus striatum of rats with nigrostriatal lesions (12). The augmentation of binding in these animals is about twice that observed in rats treated chronically with neuroleptics.

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CHAPTER 9

Dopamine Receptors, Neuroleptics, and Schizophrenia

Solomon H. Snyder

Biochemical binding techniques that successfully labeled opiate receptors (1) have permitted labeling of most neurotransmitter receptors in the brain. We will summarize applications of these methods to studies of dopamine receptors, neuroleptic action and blood levels, and abnormalities of dopamine receptors in postmortem brains of schizophrenic patients. In brief, a variety of clinical and pharmacologic evidence indicates that therapeutic actions of neuroleptics involve dopamine receptor blockade, a hypothesis that has been substantiated by direct labeling of dopamine receptors (2, 3, 4). Identification of two physically distinct dopamine receptors in the brain may facilitate development of more selective drugs for use in both psychiatric and endocrine conditions. A radioreceptor assay based on dopamine receptor binding can detect blood levels of all clinically used neuroleptics and their active metabolites and may facilitate routine monitoring of blood levels. Increased numbers of dopamine receptors in brains of schizophrenic patients appear to be related to neuroleptic drug ingestion.

The first direct biochemical investigations of dopamine receptors dealt with the dopamine-sensitive adenylate cyclase, which synthesizes cyclic AMP when stimulated selectively by dopamine (5, 6). This enzyme is enriched in parts of the brain containing dopamine and is blocked by phenothiazine neuroleptics in rough proportion to their clinical potencies.

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However, butyrophenone neuroleptics such as haloperidol, pimozide, and spiroperidol, the most potent antischizophrenic drugs, are quite weak inhibitors of this dopamine cyclase. By contrast, relative potencies of all neuroleptics including butyrophenones correlate quite closely with their affinities for dopamine receptors labeled by titrated butyrophenones such as [^3H]spiroperidol and [^3H]haloperidol (2, 3, 4). Therapeutic activity does not correlate with labeling of dopamine receptors by [^3H]dopamine itself or [^3H]apomorphine (2, 3), a dopamine-mimicking drug, since drug potencies at these binding sites resemble effects at the cyclase enzyme.

Two Types of Dopamine Receptors in the Brain

These discrepancies appear to be resolved by the demonstration of at least two distinct dopamine receptors in the brain: one, referred to as DA-1, is associated with the adenylate cyclase enzyme and labeled predominantly by the binding of [^3H]dopamine and [^3H]apomorphine; the other, referred to as DA-2, is not linked to adenylate cyclase and is labeled by [^3H]butyrophenones (7, 8, 9).

Brain lesion studies provide direct evidence for these two populations of dopamine receptors. Kainic acid, an analogue of glutamic acid, has potent excitatory effects on central nervous neurons. It destroys neurons as a result of this excitant action and thus can affect only intrinsic neurons, those whose cell bodies are located in the area in which kainic acid microinjections are made. That kainic acid injections in the corpus striatum rapidly and almost totally deplete dopamine adenylate cyclase activity indicates that DA-1 receptors are localized to intrinsic neurons (8) (Figure 9-1). These injections produce a 65%–70% loss of [^3H]apomorphine binding, suggesting that the majority of [^3H]apomorphine binding is contained on intrinsic neurons; by contrast, they produce only a 40%–50% decline in [^3H]spiroperidol or [^3H]haloperidol binding.

Where are the [^3H]spiroperidol binding sites that are resistant to kainic acid? These appear to be localized to axons and terminals of the corticostriate neuronal pathway, whose cell bodies in the cerebral cortex provide the major neuronal input to the corpus striatum. This pathway is destroyed by ablating the cerebral cortex, which depletes the residual [^3H]spiroperidol binding in the corpus striatum not affected by kainic acid lesions (8). The dopamine-sensitive adenylate cyclase, on the other hand, is not reduced by cerebral cortex ablation, which indicates that there are no DA-1 receptors on corticostriate neurons and that there is a substantial proportion of DA-2 receptors on these neurons.

The relationship of the two dopamine receptors to adenylate cyclase has been clarified by studies of guanosine triphosphate (GTP) effects. GTP reg-

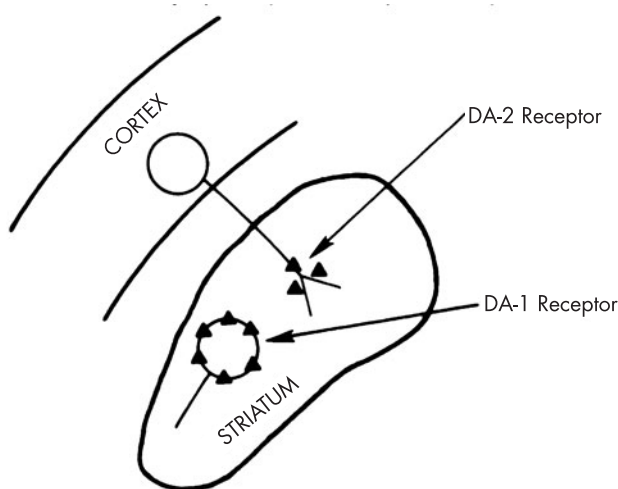


FIGURE 9-1. Two distinct postsynaptic dopamine receptors in corpus striatum.

The DA-1 receptor labeled preferentially by [3 H]apomorphine on intrinsic neurons is linked to adenylate cyclase and regulated by GTP; the DA-2 receptor labeled preferentially by [3 H]spiroperidol or [3 H]haloperidol is localized to corticostriate neurons and not linked to adenylate cyclase nor regulated by GTP.

ulates neurotransmitter receptors that are linked to adenylate cyclase in complex ways. This nucleotide decreases the affinities for the receptor of the neurotransmitter agonist or drugs mimicking it without influencing the affinity of antagonists. GTP diminishes binding of [3 H]apomorphine but not [3 H]spiroperidol, although it decreases the potency of dopamine and other agonists in competing for [3 H]spiroperidol binding in the corpus striatum (10). Kainic acid lesions abolish these effects of GTP even though substantial [3 H]spiroperidol and [3 H]apomorphine binding remains intact; however, after kainic acid lesion (which abolishes the dopamine-sensitive cyclase) the residual [3 H]spiroperidol and [3 H]apomorphine binding is no longer influenced by GTP. Since these residual DA-2 receptors are contained on the corticostriate neurons, this finding confirms that these DA-2 receptors are not linked to adenylate cyclase (9).

Distinct DA-1 and DA-2 receptors occur in all parts of the brain that contain dopamine neurons. The pituitary gland also possesses dopamine receptors. By blocking pituitary dopamine receptors, all neuroleptic drugs elevate plasma prolactin; however, by mimicking dopamine in the pituitary, bromocriptine exerts its important therapeutic effects in relieving infertility, impotence, amenorrhea, and galactorrhea associated with hypersecretion of prolactin.

The identification of two distinct dopamine receptors may have therapeutic implications. Phenothiazine neuroleptics are active at both dopamine receptors, but butyrophenones tend to be selective for DA-2 receptors. The fact that butyrophenones are quite potent in elevating serum prolactin, in causing extrapyramidal side effects, and in relieving schizophrenic symptoms indicates that all of these actions involve DA-2 receptors. Indeed, the DA-1 sites appear to be receptors in search of a therapeutic function. If DA-1 receptors are responsible for side effects such as tardive dyskinesia, more selective DA-2 drugs would be safer. Even among DA-2 receptors there may be heterogeneity; for example, we have found some differences in drug specificity between pituitary and brain DA-2 receptors (11). Chronically elevated serum prolactin has been associated with an increased incidence of mammary carcinoma. Drugs that block brain but not pituitary dopamine receptors should not elevate prolactin levels but should still reduce schizophrenic symptoms. Prospects for further subfractionating multiple populations of dopamine receptors are brightened by recent successes in differentiating multiple opiate, serotonin, α -adrenergic, and GABA receptors (9).

Dopamine Receptors in Postmortem Brains of Schizophrenic Patients

One simplistic way of viewing the dopamine hypothesis of schizophrenia is to postulate that dopamine systems are hyperactive in brains of schizophrenic patients and that this hyperactivity might be achieved by enhanced sensitivity of dopamine receptors. Several groups, including our own, have found increased [^3H]spiroperidol binding to dopamine receptors in schizophrenia (12, 13, 14, 15). However, it is possible that this increased binding is related primarily to neuroleptic drug ingestion. Chronic neuroleptic treatment of rodents increases dopamine receptor binding (16), which may account for the symptoms of tardive dyskinesia. There have been a few reports of elevated dopamine receptor binding in the brains of deceased schizophrenic patients despite the absence of drug treatment for a year or more before death (12, 13, 14). However, one of the most striking aspects of tardive dyskinesia is that in a certain percentage of patients symptoms can continue almost indefinitely after the termination of drug treatment.

In a recent study MacKay and associates (15) evaluated [^3H]spiroperidol binding to dopamine receptors in brains of schizophrenic patients as a function of drug treatment (Table 9-1). In both the caudate and nucleus accumbens the numbers of dopamine receptors were elevated only in patients receiving neuroleptics until death. Receptor levels in patients who had not received these drugs for at least 1 month before death did not differ from control values. Thus it appears likely that reported increases in dopamine re-

TABLE 9-1. Dopamine receptor binding in postmortem brains of schizophrenic patients and control subjects^a

| Brain region | Number of dopamine receptors ^b (B _{max} , pmol/g tissue) | |
|---|---|-----|
| | Mean | SE |
| Caudate | | |
| Control group (N=17) | 5.7 | 1.2 |
| Neuroleptic-treated schizophrenic group (N=21) ^c | 9.7 | 0.9 |
| Drug-free schizophrenic group (N=7) | 5.3 | 0.8 |
| Nucleus accumbens | | |
| Control group (N=16) | 4.1 | 0.4 |
| Neuroleptic-treated schizophrenic group (N=13) ^c | 6.6 | 0.5 |
| Drug-free schizophrenic group (N=4) | 3.8 | 1.3 |

^aBrain tissue was obtained from patients with a verified diagnosis of schizophrenia or from age- and sex-matched control subjects who had no history of neurologic or psychiatric disorder. The neuroleptic-treated group had received drugs up until the time of death; the drug-free group had not received neuroleptics for at least 30 days before death.

^bData refer to specific [³H]spiroperidol binding with the maximum number of binding sites (B_{max}) derived from a Scatchard analysis of saturation curves for each subject and are adapted from MacKay and associates (15).

^cSignificantly different from control group ($P<0.005$).

ceptors in brains of schizophrenic patients largely reflect neuroleptic drug effects and are not causally related to the schizophrenic process.

Since [³H]spiroperidol was used in these studies, DA-2 receptors were predominantly labeled. DA-1 receptors are not readily detected in the post-mortem human brain, and in one study (13) they did not appear to be abnormal in the brains of schizophrenic patients.

Dopamine-Based Radioreceptor Assay for Neuroleptics

In the past, neuroleptic blood levels have not been readily measured by chemical techniques. Since plasma levels are quite low, complex chemical methodology is often required and assays are not available for routine clinical application. Moreover, numerous neuroleptic drugs are used clinically, each of which requires a separate assay procedure. In addition, certain neuroleptics, such as chlorpromazine and thioridazine, may exert their therapeutic actions in part through active metabolites not measured by most chemical assays.

One direct benefit of neurotransmitter receptor binding studies has been the development of radioreceptor assays for drugs. These are analogous to radioimmunoassays. In a radioimmunoassay, one monitors the binding of a radioactive drug to an antibody raised against that drug; the drug content of the patient's plasma competes with the radioactive drug and reduces the amount bound to the antibody. In a radioreceptor assay, one measures the binding of the radioactive drug to the receptor and monitors drug content of the patient's plasma by its ability to reduce receptor binding; this approach has been applied to measurement of β -adrenergic blockers (17), tricyclic antidepressants (18), and neuroleptic levels determined by competition for [3 H]spiroperidol binding to dopamine receptors (19).

The radioreceptor assay for neuroleptics is highly specific and will detect no other drugs or endogenous substances in amounts that circulate normally. Thus, plasma samples can be used directly without purification, resulting in simplicity and speed of assay. The neuroleptic radioreceptor assay can process up to 100 samples in a morning. Since all therapeutically used neuroleptics block dopamine receptors, the assay can be used with any neuroleptic in present clinical use. Moreover, the radioreceptor assay will detect any active metabolites, since it is measuring drugs through their pharmacologic action at dopamine receptors.

Initial studies with radioreceptor assay revealed some interesting differences among neuroleptic drugs. When one measures blood levels in terms of dopamine receptor occupancy, expressed as chlorpromazine equivalents, most drugs have similar total plasma levels (Table 9-2). This is not surprising; if the drugs act by blocking dopamine receptors, therapeutic doses of different drugs should effect a similar degree of dopamine receptor blockade in the brain. If the blood-brain ratio of drug concentrations for the different neuroleptics was constant, then blood levels of all neuroleptics, measured in dopamine receptor occupancy units, should be similar.

There are some exceptions. Total plasma levels are much higher for thioridazine and mesoridazine than for other drugs. One possible explanation is that the relative penetration into the brain is less for thioridazine and mesoridazine. Blood levels after fluphenazine decanoate are quite low, much lower than after fluphenazine hydrochloride. Perhaps the constant low plasma levels occurring with fluphenazine decanoate are adequate for prophylaxis in stabilized patients, the way in which fluphenazine decanoate is used most frequently for treatment of schizophrenic patients. Alternatively, an initial rise in fluphenazine levels within the first 3 days of injection, reported by Curry and associates (24), may protect against relapse despite the subsequent extremely low levels being subtherapeutic.

Studies in patients have indicated the clinical usefulness of the radioreceptor assay (20, 21, 22, 23, 25). In one study of 30 schizophrenic patients

TABLE 9-2. Serum levels with various neuroleptics measured by radioreceptor assay^a

| Drug | Dose (mg/day) | | Neuroleptic serum level ^b (ng/mL) | |
|------------------------|---------------|-----------|--|-------------|
| | Mean | Range | Mean | Range |
| Chlorpromazine | 400 | 200–1,000 | 120 | 50–200 |
| Fluphenazine HCl | 41 | 15–16 | 120 | 60–230 |
| Fluphenazine decanoate | 45 | 10–75 | 11 | 2–25 |
| Trifluoperazine | 20 | 20 | 53 | 45–60 |
| Haloperidol | 29 | 10–50 | 111 | 70–200 |
| Molindone | 159 | 150–200 | 180 | 110–300 |
| Thiothixene | 14 | 10–25 | 155 | 130–180 |
| Thioridazine | 430 | 150–800 | 800 | 350–1,700 |
| Mesoridazine | 225 | 150–400 | 2,320 | 2,207–2,433 |

^aValues are derived from 5 to 40 patients for every drug but mesoridazine ($N=2$) and are compiled from several studies conducted in our laboratory with the neuroleptic radioreceptor assay (20, 21, 22, 23).

^bBlood levels are expressed in chlorpromazine equivalents, the concentration of chlorpromazine that would reduce [³H]spiroperidol binding to dopamine receptors to the same extent as the indicated drug.

poor clinical response was correlated with low serum level but not with low dosage of five different neuroleptics (21). Some workers using different assays have found similar results (26, 27), but others have not (28). Conceivably, measuring only the parent drug by chemical means fails to give an adequate indication of the therapeutically active drug. Plasma levels of 7-hydroxychlorpromazine correlate better with clinical response than those of chlorpromazine alone (29, 30). Could 7-hydroxychlorpromazine account in a major way for the therapeutic response to chlorpromazine? It is generally accepted that for drugs which are tightly bound to plasma protein, such as the neuroleptics, only the free drug unbound to plasma is available for penetration into the brain. Most studies have shown that 90% or more of phenothiazines in plasma are bound to protein. Recently, using fresh plasma samples, we showed that, at the blood levels which occur in clinical practice, less than 1% of total plasma chlorpromazine, thioridazine, and fluphenazine is free (31). If individuals vary in the percentage of free drug, then total plasma levels might not predict clinical response nearly so well as free levels.

In addition, active metabolites and parent drugs may vary markedly in percentage of free drug. At a concentration of 8 μ M the proportion of free 7-hydroxychlorpromazine is about 10 times greater than chlorpromazine

alone. In most studies total plasma 7-hydroxychlorpromazine levels after chlorpromazine treatment are similar to chlorpromazine levels. Thus, in chlorpromazine-treated patients free 7-hydroxychlorpromazine levels are substantially greater than those of chlorpromazine. Since 7-hydroxychlorpromazine has about the same potency as chlorpromazine in blocking dopamine receptors, the therapeutic effects of chlorpromazine may be mediated largely by 7-hydroxychlorpromazine. Similarly free levels of mesoridazine are 50 times those of thioridazine. In patients treated with thioridazine, blood levels of mesoridazine are similar to those of thioridazine; this suggests that essentially all therapeutic activity is attributable to mesoridazine, whose dopamine receptor affinity is about the same as that of thioridazine. Clearly, measuring free plasma levels of both the parent drug neuroleptic and its active metabolites may provide the ideal approach to monitoring therapeutic effects.

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Part IV

DRUG EFFECTS EXPLAINED AS ACTIONS ON NEUROTRANSMITTER RECEPTORS

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COMMENTARY

Perspectives on Simplicity and Discovery

Carol A. Tamminga

The pace and press of scientific discovery does not often allow, let alone invite, thinking back on the foundation of discoveries now commonplace. The basic process of receptor-driven research for drugs with CNS actions in brain diseases is so familiar, it is hard to imagine that it was once unknown. But in the early 1970s there was not a familiar understanding of drug targets in brain, let alone an established methodology for pursuing questions about the clinical actions of drugs at specific receptors. Receptors had not been localized in brain; they had not been isolated or molecularly defined; their pharmacology had not been associated with clinical outcomes; receptors were not visualized in human brain, nor had occupancy of receptor systems with drugs been used clinically; no cloned receptor systems existed for drug discovery.

The discoveries made by Sol Snyder and his colleagues that are illustrated in these papers have turned out to be critical to CNS drug development, while at the same time conceptually simple and eminently practical. Their brilliance and their eventual influence in drug development may not have been fully realized at the beginning. It was not easy to see that these receptor approaches would lead, on the one hand, to a widely applied methodology critical for drug development in CNS pharmacology; and, on the other hand, to the molecular characterization of receptor function, opening up new fields of receptor pharmacology in CNS research. This research profited directly from Snyder's own precept to "[develop] simple, sensitive, and specific means of measuring [small molecules]," an approach reflecting the

opinion of his famous mentor, Julie Axelrod, that “the biggest advances in science always come from new methodologies which permit measuring things simply and rapidly.” Nowhere among his many and brilliant scientific accomplishments is this approach better exemplified than in his receptor research as it underlies drug discovery.

The two pharmacology papers in this section (1, 2; Chapters 10 and 11) are illustrations of how cleverly and thoughtfully Snyder developed and applied a technique that would revolutionize the approach to drug discovery for brain diseases. The approach appears simple: Dr. Snyder and his colleagues “simply” 1) used radiochemically labeled high-affinity drugs to bind to and thereby identify a cerebral receptor, 2) correlated the affinities of various drugs at that receptor with their clinical actions in patients, 3) accepted (indeed, quantified) multiple receptor actions of a single compound (i.e., noting the complexity of drug action), and, most critically, 4) provided a “simple, sensitive, and direct assay system” for examining drug action at cerebral receptors. Most subsequent research in CNS receptor pharmacology and CNS drug development followed the exact principles Snyder and his colleagues established in these papers. These discoveries provided the methodology for receptor affinity profiling done in industry for all new drug entities, for the identification of receptor distribution in brain, and for molecular imaging of receptors in human brain and occupancy measures, as well as the basic concepts of receptor affinity–clinical response correlations in human drug actions. It is the quantification that allows comparisons across compounds and that facilitates understanding of the molecular pharmacology of their actions. One would be hard pressed to cite a more influential set of concepts and methodologies underlying modern CNS drug discovery than these.

Even after defining drug–receptor interactions, Snyder recognized the importance of correlating these receptor characteristics of a drug with their clinical action, sometimes with more than one action (i.e., to identify drug effects and to explain side effects). This association of a human drug action with its receptor affinity characteristics immediately demonstrated the clinical relevance of receptor affinity and provided the background for its important position in drug development. Moreover, Snyder understood, perhaps intuitively, that drugs have complex actions, and he went on to demonstrate this and reveal its clinical relevance. This understanding of the action of drugs at receptors is the foundation of our current approach to CNS drug action today and is used in discovery to find better drugs with fewer side effects for diseases of the brain.

Today, the process of identifying a promising compound for a CNS indication begins with characterization of the compound’s receptor affinity profile. High-throughput screening of active CNS compounds is based on

processes developed by Snyder and his colleagues (and illustrated in these papers) and then made efficient by clever engineers. Promising compounds are identified by their interesting receptor profiles (already known to be behaviorally or clinically relevant), emphasizing profiles connected with drug action and minimizing profiles associated with side effects. Once a compound gets to clinical study, its brain distribution is described by ligand binding studies and its optimal dose ranges are defined by receptor occupancy methodologies. Different dose forms (e.g., immediate vs. delayed release) have clinical actions that can be documented by distinct receptor occupancy profiles. Moreover, special characteristics of drug action (e.g., the high abuse potential of methylphenidate, correlating with fast binding and high early occupancy) can be documented by dynamic receptor occupancy and confirmed by correlations with behavioral outcome. All of these methodologies, which have come to be essential components of drug development, are based on the simple principles of receptor ligand binding developed and implemented in Snyder's laboratory in the early 1970s.

These influential basic accomplishments suggest important principles for advancing science today: think clearly and simply; focus on the critical determinants of organism or system function; identify the critical steps in cerebral processes and quantify them; develop simple methodologies for measuring important molecular, cellular, and systems behavior, and apply these broadly. But taking a keen interest in scientific discovery and risking unusual approaches must also be ingredients of Snyder's productive creativity. We are indebted to great scientific leaders who chart a course that leads them and others to broad areas of discovery. Such is the legacy of Solomon Snyder's contributions to the field of neuropharmacology and drug discovery for diseases of the brain.

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CHAPTER 10

Antischizophrenic Drugs and Brain Cholinergic Receptors

Affinity for Muscarinic Sites Predicts Extrapyramidal Effects

Solomon Snyder

David Greenberg

Henry I. Yamamura

It is generally accepted that of the phenothiazine and butyrophenone drugs commonly employed clinically, all exert fairly comparable antischizophrenic activity. Thus, a major criterion in selecting a drug is the relative frequency of side effects. Extrapyramidal actions are the most frequent and troublesome untoward effects and seem to occur to a varying degree with all phenothiazines and butyrophenones, despite extensive efforts to synthesize drugs lacking extrapyramidal effects. Because antischizophrenic and extrapyramidal effects seem inextricably linked, theories have been advanced ascribing these two actions to the same or similar mechanisms. The most heuristic of these notions is the "dopamine hypothesis" (1, 2). The dopamine theory of schizophrenia is predicated in large part on the interactions of the antischizophrenic phenothiazines and butyrophenones with brain

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catecholamines, especially dopamine. Briefly, a variety of evidence argues that the clinically efficacious phenothiazines and butyrophenones act selectively on the fundamental symptoms of schizophrenia and not simply as sedatives (3, 4). According to this reasoning, clarifying the mechanism of their antischizophrenic activity might illuminate the pathophysiology of the schizophrenic process.

Of the many biochemical effects exerted by phenothiazine drugs, very few correlate with their antischizophrenic activity. Thus, numerous biochemical actions of these drugs are produced just as effectively by the antihistaminic phenothiazine promethazine hydrochloride, which is not an efficacious antischizophrenic agent, as by chlorpromazine (5). By contrast, influences of these drugs on catecholamine metabolism indicating that they block dopamine receptors in the brain correlate closely with antischizophrenic efficacy (6, 7, 8). Recently, blockade of dopamine receptors in the brain by antischizophrenic drugs has been directly demonstrated. Specific dopamine-induced inhibition of neurons receiving dopamine terminals is antagonized by phenothiazines in proportion to their antischizophrenic activity (9). The selective stimulation of cyclic AMP formation by dopamine in areas of the brain rich in dopamine nerve terminals, thought to reflect dopamine receptor activity, is antagonized best by the antischizophrenic phenothiazines (10).

Besides accounting for their antischizophrenic effects, blockade by phenothiazines and butyrophenones of dopamine receptors can explain their extrapyramidal side effects, including akathisia, abnormal movements, and parkinsonian-like symptoms. These side effects may be analogous to abnormalities in idiopathic Parkinson disease, that follows on degeneration of dopamine neurons in the corpus striatum, a brain region responsible for motor coordination. Phenothiazines induce a functional dopamine deficiency in the corpus striatum by blocking dopamine receptors.

Besides the dopamine pathway with cell bodies in the substantia nigra and terminals in the corpus striatum, dopamine systems exist with cell bodies in the brain stem close to the interpeduncular nucleus and terminals in the olfactory tubercle and nucleus accumbens, parts of the limbic system of the brain that regulate emotional behavior (11). Recently, an extensive system of dopamine nerve terminals have been described in the cerebral cortex, especially in limbic areas (12). While most investigators agree that the extrapyramidal side effects of phenothiazines involve blockade of dopamine receptors in the corpus striatum, no definitive evidence exists regarding what dopamine pathway mediates the antischizophrenic actions of drugs.

If dopamine receptors throughout the brain respond similarly to various phenothiazines, at therapeutic antischizophrenic doses similar receptor blockade should occur in the dopamine systems. Horn and co-workers (13)

have obtained direct evidence that dopamine receptors (evaluated in terms of the dopamine sensitive adenylate cyclase) in the corpus striatum and olfactory tubercle display substantially the same responses to different phenothiazine drugs. Accordingly, with antischizophrenic doses of phenothiazines and butyrophenones, all of these drugs should exhibit the same amount of extrapyramidal side effects. While it is true that no clinically utilized antischizophrenic drug is completely free of extrapyramidal actions, there are marked differences in frequency of these effects. Butyrophenone drugs such as haloperidol (Haldol) and piperazine phenothiazines such as trifluoperazine hydrochloride (Stelazine) and perphenazine (Trilafon) produce considerably more extrapyramidal effects than alkylamino phenothiazines such as chlorpromazine (14, 15). Thioridazine hydrochloride (Mellaril), a piperidine phenothiazine, produces fewer extrapyramidal side effects than chlorpromazine, while the recently developed antischizophrenic drug clozapine manifests the lowest incidence of extrapyramidal effects of any known antischizophrenic drug (Figures 10–1 and 10–2) (16, 17, 18).

How might one account for these marked variations in frequency of extrapyramidal side effects? In general, the drugs that are more potent on a milligram basis produce more side effects. However, they are administered in correspondingly smaller doses and most schizophrenic patients are “titrated” to the optimal antischizophrenic dose. Thus, if dopamine receptor blockade, which occurs with these drugs to a similar extent at all dopamine synapses, accounts for both extrapyramidal and antischizophrenic actions, the more potent antischizophrenic drugs should display the same incidence of extrapyramidal effects as the weaker ones. Some authors have suggested that the low incidence of these side effects occurring with clozapine can be explained by data suggesting that dopamine receptor blockade by this drug is more “surmountable” than with most phenothiazines (19). A simpler and more drastic explanation is that the antischizophrenic effects of drugs are not produced by dopamine receptor blockade and that the dopamine hypothesis of schizophrenia is invalid or at least severely deficient.

An alternative explanation for variations in the incidence of extrapyramidal effects lies in the anticholinergic actions of phenothiazines that might, to varying degrees, attenuate extrapyramidal effects. Phenothiazines do bear structural resemblances to drugs, such as atropine sulfate, which block muscarinic cholinergic synapses. Indeed, “belladonna-like” actions such as dry mouth, dizziness, and difficulty in urination are frequently complained of side effects of the phenothiazines. Since Charcot’s introduction of belladonna in the therapy of Parkinson disease, antagonists of muscarinic cholinergic receptors have been major drugs in the treatment of this condition. Stille et al. (20) observed that clozapine was a fairly potent antagonist of acetylcholine actions on the guinea pig ileum and suggested, as did Anden and

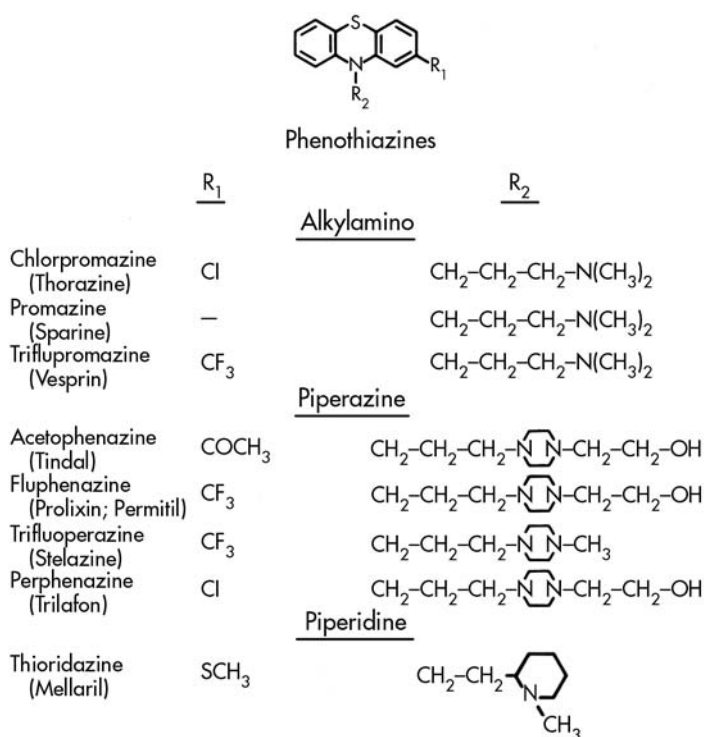
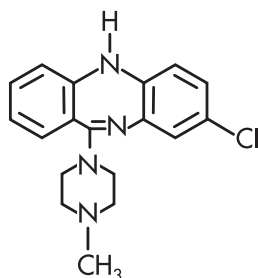


FIGURE 10-1. Structures of phenothiazines.

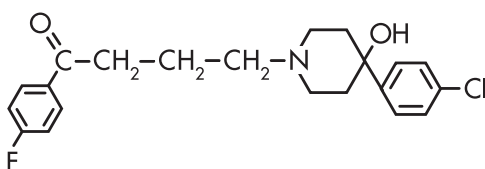
Stock (21), that its relative paucity of extrapyramidal effects might be related to its anticholinergic activity.

We speculate that by blocking muscarinic cholinergic receptors in the brain, phenothiazines might antagonize their own tendency to elicit extrapyramidal symptoms via dopamine receptor blockade. Accordingly, we predict that the potency of phenothiazines and butyrophenones as antagonists of muscarinic cholinergic receptors in the brain should vary inversely with their tendency to evoke extrapyramidal symptoms. For instance, perhaps trifluoperazine elicits a high incidence of these side effects, because it is a very weak muscarinic anticholinergic and thus is unable to counteract its own propensity to produce extrapyramidal effects. Clozapine would be expected to be the most potent muscarinic anticholinergic; thioridazine should be highly effective but less so than clozapine; while chlorpromazine should be a better anticholinergic than piperazine drugs such as trifluoperazine but less potent than thioridazine.

To evaluate this hypothesis, one must be able to quantify affinity for muscarinic cholinergic receptors in the brain. Peripheral anticholinergic ef-

Dibenzodiazepine

Clozapine

Butyrophenone

Haloperidol

FIGURE 10-2. Structures of clozapine and haloperidol, examples respectively of dibenzodiazepine and butyrophenone classes.

fects are readily measured on smooth muscle by traditional pharmacological methods, but these may not correspond precisely to relative affinities in the brain. Recently, techniques have been developed to identify biochemically the brain's muscarinic cholinergic receptor (22, 23, 24). We have measured the binding of 3-quinuclidinyl benzilate (QNB), a potent antagonist of muscarinic cholinergic receptors, to membrane preparations from the central nervous system (23, 24). The binding of highly radioactive QNB represents an almost exclusive interaction with muscarinic cholinergic receptors. Muscarinic antagonists, such as atropine and scopolamine hydrobromide, displace specific QNB binding with a very high affinity of about 10^{-9} M. Acetylcholine and drugs that mimic acetylcholine have affinities for these binding sites that parallel their known ability to mimic acetylcholine. For instance, oxotremorine, which is considerably more potent in stimulating muscarinic cholinergic receptors than acetylcholine, pilocarpine hydrochloride, or carbamylcholine, possesses 10 times or more affinity for QNB binding sites than these other drugs (23). Moreover, drugs that are known to stimulate or block nicotinic but not muscarinic cholinergic receptors have negligible affinity for QNB binding sites. In addition, numerous noncholinergic drugs do not bind to the QNB sites. Specific QNB binding largely parallels the distribution of muscarinic cholinergic receptors throughout the brain as determined by neurophysiologic techniques.

Possessing a simple, sensitive, and specific assay for the muscarinic cholinergic receptor in the brain, we evaluated the relative affinities of a variety of antischizophrenic drugs (Table 10-1) (25). Their affinity for the muscarinic receptor in whole brain correlates inversely in an impressive fashion with their tendency to elicit extrapyramidal side effects. Clozapine, which is almost devoid of these side effects, has the greatest affinity for QNB binding

TABLE 10-1. Relative affinities of phenothiazines and butyrophenones for muscarinic cholinergic receptor binding in brain correlates inversely with extrapyramidal side effects

| Drug class | ED ₅₀ concentration, M ^a | Relative affinity for muscarinic receptor | Frequency of extrapyramidal side effects rank by class (1= most side effects) |
|------------------|--|---|---|
| Dibenzodiazepine | | | |
| Clozapine | 2.6×10 ⁻⁸ | 385 | 5 |
| Piperidine | | | |
| Phenothiazine | | | |
| Thioridazine | 1.5×10 ⁻⁷ | 66.7 | 4 |
| Alkylamino | | | |
| Phenothiazine | | | |
| Promazine | 6.5×10 ⁻⁷ | 15.2 | 3 |
| Chlorpromazine | 1.0×10 ⁻⁶ | 10.0 | |
| Triflupromazine | 1.0×10 ⁻⁶ | 10.0 | |
| Piperazine | | | |
| Phenothiazine | | | |
| Acetophenazine | 1.0×10 ⁻⁵ | 0.90 | 2 |
| Perphenazine | 1.1×10 ⁻⁵ | 0.91 | |
| Trifluoperazine | 1.3×10 ⁻⁵ | 0.78 | |
| Fluphenazine | 1.2×10 ⁻⁵ | 0.83 | |
| Butyrophenone | | | |
| Haloperidol | 4.8×10 ⁻⁵ | 0.21 | 1 |

^aAffinity for the muscarinic receptor is defined as the reciprocal×10⁻⁵ of the ED₅₀ value, defined as the molar concentration of drug that displaced by 50% the specific binding of tritiated QNB (1 nmol) to whole rat brain homogenates. ED₅₀ values were obtained by log-probit plots of the effects of four concentrations of each drug assayed in triplicate. Each experiment was replicated twice.

sites with a potency similar to standard anticholinergic antiparkinsonian drugs such as benzotropine mesylate (Cogentin) and trihexyphenidyl hydrochloride (Artane). Thioridazine, which next to clozapine elicits the fewest extrapyramidal symptoms, displays the second greatest affinity for muscarinic sites in the brain, about one sixth that of clozapine. The alkylamino phenothiazines promazine hydrochloride, chlorpromazine, and triflupromazine hydrochloride, whose moderate incidence of extrapyramidal actions is greater than that of thioridazine, have correspondingly less affinity for QNB binding sites. The piperazine phenothiazines, acetophenazine maleate, fluphenazine

hydrochloride (Prolixin), trifluoperazine hydrochloride, and perphenazine (Trilafon), which produce considerably more extrapyramidal side effects than chlorpromazine, are one tenth to one fifteenth as effective in binding to the muscarinic receptors. Similarly, the butyrophenone haloperidol, whose frequency of extrapyramidal effects is as high or higher than the piperazine phenothiazines, has very little affinity for the muscarinic receptor.

The apparent balance of cholinergic and dopaminergic mechanisms in producing extrapyramidal symptoms is presumed to operate in the corpus striatum. Our initial experiments employed the whole brain. However, in the corpus striatum of the rat, the relative affinities of clozapine, thioridazine, chlorpromazine, and trifluoperazine for QNB binding sites are the same as in the whole brain. To determine if findings in rat brain are applicable to primates, we measured the affinities of clozapine, thioridazine, chlorpromazine, and trifluoperazine for the muscarinic receptor site in the corpus striatum of rhesus monkey (Table 10–2). The relative affinities of these four drugs in monkey putamen (part of the corpus striatum) are the same as in the rat corpus striatum and whole brain. We wondered whether the muscarinic receptor site in the periphery would have similar properties as in the brain. We have demonstrated specific QNB binding to muscarinic cholinergic receptors in the guinea pig ileum. The relative affinities of clozapine, chlorpromazine, and trifluoperazine appear to be fairly similar in the guinea pig ileum as in the brain (Table 10–2).

The hypothesis linking anticholinergic activity and extrapyramidal side effects of antischizophrenic drugs requires that relative affinities for the dopamine and muscarinic cholinergic receptors not parallel each other. For instance, if trifluoperazine had only one one hundredth the affinity of thioridazine for both dopamine and acetylcholine receptors, the actions on these two transmitter systems would balance each other out and the two drugs should elicit extrapyramidal effects to the same extent. Miller and Iversen (26) have measured the relative potency of a variety of phenothiazines and butyrophenones in antagonizing the dopamine-sensitive adenylate cyclase in the corpus striatum of rat brain. Apparent blockade of dopamine receptors does not correlate at all with affinities for the muscarinic cholinergic receptor. Thus, trifluoperazine is almost 10 times as potent in blocking the dopamine-sensitive adenylate cyclase as thioridazine, while it is only one sixtieth as active in blocking the cholinergic receptor site. This suggests that therapeutic antischizophrenic dopamine receptor blockade requires almost 10 times higher brain levels of thioridazine than of trifluoperazine. At these higher brain levels, thioridazine should block cholinergic receptors, hence alleviate extrapyramidal symptoms, even more than at equimolar concentrations.

This model provides a simple system to screen drugs for extrapyramidal side effects. One simply measures the affinity of agents for dopamine and

TABLE 10-2. Affinity of phenothiazines for muscarinic cholinergic binding sites in corpus striatum and guinea pig ileum

| | Relative affinity for QNB binding sites ^a | | |
|-----------------|--|----------------|------------------|
| | Rat corpus striatum | Monkey putamen | Guinea pig ileum |
| Clozapine | 410 | 378 | 111 |
| Thioridazine | 94.2 | 82.5 | — |
| Chlorpromazine | 14.3 | 11.6 | 12.5 |
| Trifluoperazine | 1.35 | 1.03 | 2.17 |

^aAffinity for muscarinic cholinergic receptors, represented by specific tritiated QNB binding, is defined as the reciprocal $\times 10^{-5}$ of the molar concentration that displaces by 50% the specific binding of tritiated QNB (1 nmol) to homogenates of rat corpus striatum, monkey putamen, or guinea pig ileum. Displacement percentages were obtained by log-probit plots of the effects of three concentrations of each drug assayed in triplicate. The procedure for homogenization and assay was essentially the same as described for whole brain (23) except for the guinea pig ileum that was minced prior to homogenization with a conical glass pestle followed by Polytron treatment.

muscarinic receptors. The most active central muscarinic cholinergics should elicit fewest extrapyramidal side effects with no attenuation of antischizophrenic activity. The ideal drug would be considerably more active in blocking brain than peripheral cholinergic receptors, and therefore provoke fewer peripheral dopamine-like actions such as dry mouth and urinary retention.

Bunney and Aghajanian (written communication) recently observed that all antischizophrenic phenothiazines accelerate the firing of dopamine cells in the A10 grouping that projects to the limbic system, while the firing of cells in the substantia nigra (that projects to the corpus striatum) could be accelerated only by phenothiazines that elicit prominent extrapyramidal side effects. Whether these varying influences on the two dopamine cell groups relate to differential anticholinergic actions of the drugs in the corpus striatum is unclear.

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CHAPTER 11

Tricyclic Antidepressants

Therapeutic Properties and Affinity for α -Noradrenergic Receptor Binding Sites in the Brain

David C. U'Prichard

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Solomon H. Snyder

Tricyclic antidepressants, the major agents used in treating depression, vary considerably in their therapeutic properties. Tertiary amine tricyclics are particularly effective in patients with psychomotor agitation and also elicit the highest incidence of sedative and hypotensive side effects, whereas the secondary amines are more effective in retarded depression since they produce psychomotor activation and are less likely to elicit sedative and hypotensive side effects (1, 2, 3, 4). Since the tertiary amine tricyclics are more potent inhibitors of serotonin uptake than the secondary amines, while the reverse is true for the inhibition of norepinephrine uptake, some investigators have suggested that psychomotor activation by tricyclic antidepressants results from blockade of norepinephrine uptake in the central nervous sys-

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tem, while reduction of agitation is attributable to inhibition of serotonin uptake (4, 5, 6, 7).

α -Noradrenergic receptor sites in the brain can be labeled with several tritiated drugs (8, 9). The sedative-hypotensive actions of neuroleptics are predicted by their affinities for α -receptors, suggesting that these agents cause sedation and hypotension by blocking α -noradrenergic receptors (10). We now report a close correlation between the ability of tricyclic antidepressants to reduce psychomotor agitation and their affinities for α -noradrenergic binding sites in brain membranes.

α -Receptor binding assays with ^3H -labeled WB-4101 (2-([2',6'-dimethoxy]-phenoxyethylamino) methylbenzodioxan), a potent α -adrenergic antagonist, were performed essentially as described (9). Homogenates (Brinkmann Polytron) of fresh rat whole brain excluding the cerebellum in Tris-HCl buffer were washed twice by centrifugation, and then suspended in 50 volumes of cold 50 mM Tris-HCl buffer, pH 7.7, at 25°C. For binding assays, each tube received 1.0 mL of membrane suspension (20 mg, wet weight), 0.2 nM [^3H]WB-4101 (11.7 c/mmole; custom tritiated, New England Nuclear, more than 95% pure), and various concentrations of nonradioactive drugs. The incubation volume in each tube was brought to 2 mL with cold 50 mM Tris-HCl buffer, pH 7.7, at 25°C. Triplicate tubes were incubated at 25°C for 20 minutes and rapidly filtered under vacuum through Whatman GF/B filters with two 5-mL rinses of cold buffer. The filters were counted by liquid scintillation spectrometry in 10 mL of Formula 947 (New England Nuclear) at 37% efficiency.

Specific binding of [^3H]WB-4101 was measured as the excess over blanks taken in the presence of 100 μM (–)-norepinephrine. Under these conditions, specific binding (600 to 700 count/min) constituted two-thirds of the total binding. Specific binding of [^3H]WB-4101 to rat brain membranes is saturable, with a dissociation constant (K_D) of about 0.5 nM.

The tertiary amine tricyclics in general are more potent than the secondary amines in competing for [^3H]WB-4101 binding to α -noradrenergic receptors (Table 11–1). Among the tertiary amines, doxepin and amitriptyline are equally potent and about 2.5 times more potent than chlorimipramine and imipramine. With apparent inhibition constant (K_i) values of 23 to 24 nM, doxepin and amitriptyline are only six times weaker than the potent classical α -receptor antagonists phentolamine and phenoxybenzamine (9). The secondary amines are only one-third as potent as the corresponding tertiary amines in competing for [^3H]WB-4101 binding. The tertiary amine tricyclics are substantially more potent in competing for α -receptor binding than in blocking norepinephrine and serotonin uptake in brain slices and synaptosomes (6).

TABLE 11-1. Tricyclic antidepressants: comparison of affinities for [3 H]WB-4101 binding sites with *in vivo* pharmacological properties

| Drug | Inhibition of [3 H]WB-4101 binding, K_i (nM) | Ability to cause psychomotor activation | Sedative and hypotensive properties | Ability to increase arousal threshold in rabbits |
|-------------------------|--|---|-------------------------------------|--|
| Tertiary amines | | | | |
| Doxepin | 23 \pm 5 | — | ++ | |
| Amitriptyline | 24 \pm 4 | — | ++ | 1.0 |
| Chlorimipramine | 55 \pm 11 | — | + | 1.1 |
| Imipramine | 58 \pm 5 | + | + | 1.2 |
| Secondary amines | | | | |
| Nortriptyline | 71 \pm 10 | ++ | + | 1.3 |
| Desipramine | 148 \pm 18 | +++ | — | >2.0 |
| Protriptyline | 277 \pm 24 | +++ | — | 2.0 |

Note. For each drug, inhibition of [3 H]WB-4101 binding was measured at three to six concentrations, and the median inhibition concentrations (IC_{50}) were determined by log-probit analysis. These were converted to apparent K_i values by the equation $K_i = IC_{50} / [1 + ([^3H]WB-4101)] / K_D$, where K_D is the dissociation constant of [3 H]WB-4101, 0.5 nM. Each value is the mean \pm standard error of four experiments, each conducted in triplicate. The qualitative data in columns 2 and 3 were derived from reviews of clinical and animal studies (1, 2, 3, 4, 7, 14). In animals, sedation and psychomotor activation were measured in terms of alteration of barbiturate-induced sleep, cortical electroencephalogram, spontaneous motor activity, and drowsiness; hypotensive potential was demonstrated as decreases in blood pressure and heart rate after systemic administration, and as blocking ability in isolated aortic and papillary muscle preparations. Clinical data correlate reduction in insomnia and agitation, and general hypnotic effects, with sedative potential, increased restlessness with psychomotor activation, and incidence of orthostatic hypotension with hypotensive potential. The lack of quantitative data obtained in the same experimental conditions over the spectrum of antidepressants did not permit statistical weighing of the data for averaging, and hence quantitative comparisons could not be made. The data in column 4 for six antidepressant drugs were obtained from Poeldinger (2); they represent the relative doses of drugs (amitriptyline=1.0) needed to raise the threshold of the arousal reaction in the rabbit, after electrical stimulation of the mesencephalic reticular formation, to 150% of control value.

The secondary amine tricyclics, especially desipramine and protriptyline, tend to cause psychomotor activation in patients and are particularly useful in treating retarded depression, whereas the tertiary amines, with the possible exception of imipramine, cause little psychomotor activation (2, 3, 7). The relative psychomotor activating effects of these drugs correlate inversely with their potency in competing for α -noradrenergic binding sites since desipramine and protriptyline are the weakest α -blockers and the tertiary amines are substantially more active. Tricyclic drugs that fail to cause psychomotor activation are more sedating and have greater therapeutic utility in relieving the psychomotor agitation of depressed patients (2).

Although it is difficult to distinguish rigorously between the relative activities of these drugs in eliciting particular clinical effects, some quantitative evaluations have been performed (2). For six tricyclic antidepressants, selective therapeutic efficacies for depressive psychomotor agitation versus psychomotor inhibition correlate well with affinities for α -noradrenergic binding sites (11) (Figure 11-1). Inhibition by these drugs of the arousal reaction in rabbits after electrical stimulation of the midbrain reticular formation provides an animal model for clinical reduction of psychomotor agitation (2). The rank-order of potencies of tricyclic drugs in eliciting this effect parallels their affinities for α -receptor sites with a correlation coefficient of 0.94 ($P < 0.05$) (Table 11-1).

Earlier we demonstrated that the binding to brain membranes of [^3H]WB-4101 represents a specific association with α -noradrenergic receptor sites (9). The suggestion that the effects of the tricyclic antidepressant drugs on these binding sites represent blockade of functional α -receptors is supported by findings that the tertiary amine tricyclic drugs are more potent than the secondary amine drugs in blocking norepinephrine stimulation of cyclic AMP (adenosine 3',5'-monophosphate) accumulation in slices of rat cerebral cortex (12).

To explain different therapeutic effects of various tricyclic antidepressants it has been suggested that secondary amines such as desipramine and protriptyline elicit psychomotor activation and enhance "drive" by blocking norepinephrine uptake, while tertiary amines selectively elevate mood by inhibiting neuronal uptake of serotonin (4, 5, 6, 7). According to this model the anti-agitation actions of the tertiary amines are presumably associated with inhibition of serotonin uptake. Alternatively, the intrinsic antidepressant efficacies of all the tricyclic drugs may be similar and related to inhibition of neuronal uptake of one or another (or both) of the biogenic amines. Differential effects on psychomotor agitation and sedation-hypotension may be related to relative α -noradrenergic blocking actions. In the absence of α -noradrenergic blocking activity, all the tricyclic antidepressants may cause a similar degree of psychomotor activation. However, the α -blocking

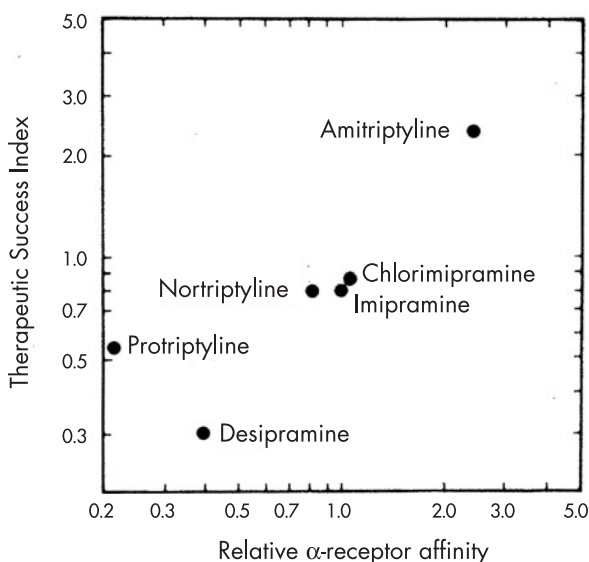


FIGURE 11-1. Tricyclic antidepressants: correlation between affinities for [3 H]WB-4101 α -noradrenergic binding sites in the rat brain, and relative therapeutic efficacies in relief of depressions with psychomotor agitation compared to relief of depressions with psychomotor inhibition, calculated from available data (2, 11).

The α -receptor affinity values were calculated from the reciprocals of the K_i values shown in Table 11-1, giving imipramine an arbitrary affinity value of 1.0. The correlation coefficient $r=0.84$ is significant at $P<0.05$. If protriptyline, which, unlike the other tricyclic antidepressants in the figure, is lacking a middle ring, is excluded, the correlation coefficient $r=0.99$ is significant at $P<0.001$, and the slope is 1.1. The therapeutic success index is defined in (11).

properties of the tertiary amines would counteract such effects and also confer a capacity to relieve agitation and to cause sedation and hypotension. The differential effects of tertiary and secondary amine tricyclics may be modified to a certain extent because of demethylation of the tertiary amine drugs, which occurs to a certain extent in most patients. The relative specificity of the tricyclic antidepressants as α -receptor antagonists is indicated by the fact that they are about 3,000-fold weaker inhibitors of the binding of dopamine and β -adrenergic ligands, respectively (13).

Certain neuroleptics are even more active in eliciting sedation and hypotension than the tertiary amine tricyclics. Earlier we observed a close association between the relative sedative-hypotensive actions of neuroleptics and their affinities for α -noradrenergic receptor binding sites labeled with

[³H]WB-4101 (10). The neuroleptics with greatest sedative properties are substantially more potent in their effects on α -noradrenergic receptor sites than any of the tricyclic drugs examined there (10). Interestingly, thioridazine, a phenothiazine neuroleptic whose relative α -noradrenergic blocking activity is among the greatest of all neuroleptics examined (10), is efficacious in relieving agitated depression (2). It is possible that its utility in treating agitated depression may derive from α -noradrenergic blocking effects in the brain and may therefore represent a mechanism of action similar to that of the anti-agitation actions of tertiary amine tricyclic antidepressants such as amitriptyline and doxepin.

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11. The therapeutic efficacies, derived from clinical studies of 40 to 100 patients taking each individual antidepressant (2), are based on the proportion of therapeutic successes for each drug in the two depressive syndromes—agitated depression and retarded depression. The ratio figure for each drug, obtained by dividing the proportionate, or percent, success in treating patients with agitated depression, by the proportionate, or percent, success in treating patients with retarded depression, is called the “therapeutic success index” for that particular drug. A drug with an index of 3.0 would be three times more successful in treating patients with agitated depression than in treating patients with retarded depression.
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Part V

DRUG ACTIONS AND SEROTONIN RECEPTOR SUBTYPES

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COMMENTARY

Clinical Data Stimulation of Basic Research: The Far-Reaching Clinical Significance of Serotonin Receptor Subtype Identification

Herbert Y. Meltzer

Along with a passionate interest in drug action in relation to receptor subtypes—the one facet of Sol Snyder's incredibly diverse research contributions that is the subject of this commentary—I am proud to note that Sol and I share many other things in common. In addition to some of the familial and ethnic characteristics Sol describes in the introduction to this volume of his major contributions to neuroscience, they include 1) a serious flirtation with philosophy before discovering natural science; 2) an intense, enduring attraction and commitment to neurochemistry and neuropsychopharmacology, beginning immediately at first contact; 3) a position as a Research Associate in the Laboratory of Clinical Science at NIMH; 4) being a neighbor of Pedro Cuatrecasas, who developed the core methodology for ligand binding (in my case, we lived next door to each other); and 5) a passionate interest in music. Where we diverge most is that Sol rarely wavered, to my knowledge, from his total personal commitment to “the bench” (basic science) and I felt drawn to study “the bedside” (clinical research) as well as basic research. Clearly his focus produced great basic science that others were able to translate into the clinical arena.

The two *Science* papers of Sol's on which I have been asked to comment have shaped much of my basic and clinical research and that of many others in our field. These two studies, the first on the effect of subchronic antide-

pressant treatment on serotonin (5-HT) receptor binding (1; Chapter 12) and the second on the identification of 5-HT₁ and 5-HT₂ receptors (2; Chapter 13), appeared in 1980–1981, a time when interest in distinguishing serotonin receptor subtypes and understanding the mechanism of action of antidepressant drugs attracted many, including me, to the nascent fields of psychopharmacology and neuroscience. The importance of understanding the role of serotonin in controlling behavior at the molecular, cellular, and behavioral levels was already appreciated, but in fact, very little was known. These two papers made seminal contributions to this end and thus were enormously generative of future research, even if they did not solve the still controversial puzzle of just how antidepressant drugs do work.

The paradigm that Snyder and Steve Peroutka applied in these two classic papers was one that Snyder and other colleagues had previously used to advantage with regard to another key neurotransmitter, dopamine; another class of drugs, the antipsychotic drugs; and another major psychiatric disorder, schizophrenia (3). The paradigm had a number of key steps. The process, in this case, included showing 1) that drugs having some serotonergic action, either as agonist or antagonist, had different affinities for two types of putative serotonin receptors, identified by binding to two very different types of ligands—[³H]-N-methylspiperone (5-HT₂) or [³H]-5-HT (5-HT₁); 2) that biochemical assay (inhibition of serotonin-sensitive adenylate cyclase) correlated with 5-HT₂ but not 5-HT₁ ligand binding; 3) that antagonism of head twitches correlated with affinity for the 5-HT₂ and not the 5-HT₁ receptors; 4) that receptor-selective agonists had either excitatory (5-HT₂) or inhibitory (5-HT₁) effects at 5-HT synapses; and 5) that only one of nine drugs they studied had similar affinities for the two receptors.

Thus, these two papers from the Snyder laboratory quite clearly establish that there were at least two subclasses of serotonin receptors, then called 5-HT₁ and 5-HT₂ (which we now know include multiple subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, and 5-HT_{2C} receptors); that the antidepressants, with the exception of fluoxetine, have a high affinity for the 5-HT₂ but not the 5-HT₁ receptor; that the 5-HT₁ receptor is, and the 5-HT₂ receptor is not, coupled to adenylate cyclase, and that the rat head twitch response is mediated by stimulation of the 5-HT₂ and not the 5-HT₁ receptor. At approximately the same time, Josee Leysen, of the Janssen laboratories, also distinguished between these two types of serotonin receptors, using similar binding methods (4).

The information on serotonin receptor subtyping initiated by these studies has been enormously important to neuropsychopharmacology, physiology, and genetics. Drugs for schizophrenia, depression, anxiety, sleep, and cognition have been and are being developed based upon knowledge of serotonin receptor subtypes. This knowledge has also informed our understanding of many side effects—such as weight gain, nausea and vomiting,

sedation, and extrapyramidal symptoms—of many drugs with a serotonergic component. My research on the role of 5-HT_{2A} receptor antagonism in the action of atypical antipsychotic drugs, which played a key role in the development of the current group of drugs used to treat schizophrenia, bipolar disorder, drug-induced psychoses, and senile psychoses, was strongly influenced by the discovery of the 5-HT₂ receptor by Snyder, Leysen, and colleagues (4). Interestingly, Peroutka and Snyder might have made the discovery of the importance of 5-HT_{2A} receptor antagonism to the action of clozapine had they related its ED₅₀ for blocking rat head twitch (Chapter 13 [2], Figure 13–1A) to the ED₅₀ for blockade of apomorphine-induced stereotypy, which they reported for haloperidol, chlorpromazine, and pipamperone in their earlier paper (3). They noted that these three neuroleptics were potent antagonists of the serotonin syndrome despite having markedly different potencies as dopamine receptor blockers. As shown in Figure 13–1A, clozapine was comparable to pipamperone and chlorpromazine as a 5-HT₂ antagonist, but with its ED₅₀ for blocking apomorphine-induced stereotypy so much higher (3), the 5-HT_{2A}/D₂ ratio of clozapine far exceeded that of haloperidol, chlorpromazine, and pipamperone and might have suggested the 5-HT_{2A}/D₂ hypothesis of atypical antipsychotic drugs that I advanced (5). Interestingly, the high affinity of clozapine for the 5-HT₁ receptor compared to the other antipsychotic drugs is also apparent in their study (1). This, too, has become of very great importance in understanding the mode of action of clozapine and the development of new antipsychotic drugs (6).

It is worth noting that prerequisite to applying the paradigm outlined above was valid clinical data and that Sol Snyder, because of his clinical training as a psychiatrist, was better able to evaluate and utilize that information than were nonclinician pharmacologists. I point this out because although translational research is one of the buzzwords of the day, far too little credit is given to those who provide the clinical data that laboratory scientists can use to test hypotheses, and, as I noted above, this is one of the areas where I have been privileged to contribute. Thus, before these two studies of Snyder and colleagues could have been conceived, it was essential for clinicians to have identified effective and noneffective therapies for depression and schizophrenia, since both these papers (1, 2) and the prior study on antipsychotic drugs and dopamine (3) are dependent on the clinical data that had been reported in the two decades prior to these publications. Clinicians first had to identify drugs like imipramine, iproniazid, and fluoxetine as antidepressants, but not antipsychotics, and chlorpromazine, haloperidol, and clozapine as antipsychotics, but not antidepressants. Knowledge of the time course of the action of the first group of drugs as antidepressants was also essential to the paradigm employed, which focused on the delayed effects of the drug, not the immediate set. Did they work immediately, as for example,

diazepam did as an anxiolytic, or was the onset of action delayed for weeks, as is the case with antidepressants? These were not easy issues to get correct answers to. In the 1970s, clinical psychopharmacology, though blessed by people of great skill, was far from fully developed. The art and science of clinical testing was in its infancy. Many will argue that even today it is fraught with error. The possibility that there might be a subgroup of the antidepressant drugs under investigation that did not show the expected relationship to the (serotonin) receptor being studied, because they had a distinct mechanism of action, was not considered. In the case of the antidepressants and the 5-HT₁ and 5-HT₂ receptor hypothesis that Peroutka and Snyder developed, the most important drug was fluoxetine (Prozac), which did *not* show the high affinity for the 5-HT₂ receptor that the other antidepressants did. Confronted with the potentially fatal inconsistency with their hypothesis that antidepressants acted via their effects on 5-HT₂ receptors, Peroutka and Snyder offered the same defense that Fridolin Sulser did when confronted with the exceptional behavior of fluoxetine with regard to his hypothesis that beta-adrenergic receptor downregulation was the holy grail of antidepressant action (7)—namely, that fluoxetine was questionably effective. Living as we do in an era in which the efficacy of all antidepressants has been questioned, one can easily understand why Peroutka and Snyder chose this option!

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CHAPTER 12

Long-Term Antidepressant Treatment Decreases Spiroperidol-Labeled Serotonin Receptor Binding

Stephen J. Peroutka
Solomon H. Snyder

The behavioral depression following biogenic amine depletion by reserpine in animals and man and the potentiation of amine action by antidepressants suggest a functional deficiency of biogenic amines in depressive disorders (1). Although the actions of norepinephrine and serotonin are potentiated by tricyclic antidepressants and monoamine oxidase (MAO) inhibitors, these effects occur immediately, whereas there is a 1- to 3-week lag before therapeutic response (2). Moreover, drugs such as cocaine block the uptake of amines but are not clinically effective antidepressants (3), and drugs such as iprindole relieve depression but do not inhibit amine uptake (4). Antidepressant agents are also fairly potent blockers of muscarinic cholinergic (5), α -adrenergic (6), histamine (7), and serotonin (8) receptors. However, receptor blockade also occurs immediately and thus does not correlate with the time course of therapeutic responses. In contrast, long-term, but not short-term, treatment with antidepressants reduces the activity of a norepinephrine-stimulated adenylate cyclase in brain tissue (9) and reduces

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the number of β -adrenergic receptor sites in brain membranes (10, 11, 12). We now report that long-term treatment with tricyclic antidepressants, the atypical antidepressant iprindole, and the MAO inhibitor pargyline decreases the number of serotonin receptors labeled by [3 H]spiroperidol.

Male Sprague-Dawley rats (6 weeks old, weighing 150 to 175 g) were given daily 0.5-mL intraperitoneal injections of various drugs or saline for 21 days. Doses of all drugs were 10 mg/kg, except for pargyline (25 mg/kg). Twenty-four hours after the final injection, rats were decapitated; the brains were removed, dissected over ice, and stored at -70°C until assay. For studies of drug competition, the fresh brains of rats that had not been given injections were used immediately for assay. The frontal cerebral cortex was used for all of the binding studies except the binding of [3 H]spiroperidol to dopamine receptors, for which the corpus striatum was used. The tissues were homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 700g for 10 minutes. The P_1 fraction was discarded and the supernatant was centrifuged at 20,000g for 10 minutes; the sediment was suspended in Tris-HCl buffer (pH 7.7 at 25°C) and centrifuged again at 20,000g for 10 minutes. The sediment was then suspended in 80 volumes of Tris-HCl buffer, and the tissue was immediately used in binding assays. Briefly, 0.1 mL of ^3H -labeled ligand, 0.1 mL of displacing drug, and 0.8 mL of tissue suspension were incubated for varying periods (13, 14). The reaction was terminated by rapid filtration under vacuum through Whatman GF/B filters, which were washed three times with 5 mL of Tris-HCl buffer. The labeled material retained on the filters was counted by liquid scintillation spectrometry.

The antidepressants examined compete for several receptor binding sites (Table 12-1). In general, the greatest potencies occur at histamine H_1 receptors labeled by [3 H]mepyramine. However, relative potencies of drugs at these binding sites do not correlate with clinical potencies; amitriptyline is used clinically at dosages similar to those of desipramine, but at histamine H_1 receptors, it is 75 times more potent. The antidepressants are fairly potent at muscarinic cholinergic receptors labeled by [3 H]quinuclidinylbenzilate ([3 H]QNB), which correlates well with clinical anticholinergic side effects (5). Relative potencies of tricyclic antidepressants at α -adrenergic receptors labeled by [3 H]WB-4101 (2-([2,6-dimethoxy]phenoxyethylamino)-methylbenzodioxan) are similar to effects at muscarinic receptors and correlate with sedation and relief of psychomotor agitation (6). Two distinct populations of serotonin receptors are labeled, one with [3 H]serotonin and the other with [3 H]spiroperidol; [3 H]lysergic acid diethylamide ([3 H]LSD) labels both sites to the same extent (14). Antidepressants are much more potent at the [3 H]spiroperidol-labeled receptors (serotonin-2) than at the [3 H]serotonin-labeled receptors (serotonin-1), effects on [3 H]LSD binding being intermediate. The antidepressants are substantially less potent at dopamine receptors

TABLE 12-1. Antidepressant affinities (K_i) for neurotransmitter receptor binding sites

| Drug | K_i (nM) | | | | | | | |
|---------------------------------|--|---|--|-------------------------------------|--|---|--|---------------------------------------|
| | Histamine H_1 ([3H]mepyramine) | Muscarinic cholinergic ([3H]QNB) | α -Adrenergic ([3H]WB-4101) | Serotonin-1 ([3H]serotonin) | Serotonin-1 + serotonin-2 ([3H]LSD) | Serotonin-2 ([3H]spiro- peridol) | Dopamine ([3H]spiro- peridol) | β -Adrenergic ([3H]DHA) |
| Tertiary amines | | | | | | | | |
| Amitriptyline | 3.2 \pm 0.6 | 11 \pm 2.1 | 22 \pm 4.0 | 1,700 \pm 70 | 170 \pm 60 | 13 \pm 2.8 | 290 \pm 77 | 6,800 \pm 360 |
| Imipramine | 18 \pm 3.9 | 89 \pm 20 | 54 \pm 17 | 10,000 \pm 3,400 | 1,800 \pm 600 | 245 \pm 69 | 610 \pm 170 | 38,000 \pm 2,000 |
| Secondary amines | | | | | | | | |
| Desipramine | 240 \pm 19 | 210 \pm 37 | 130 \pm 29 | 9,500 \pm 3,000 | 3,200 \pm 420 | 540 \pm 190 | 980 \pm 270 | 4,200 \pm 95 |
| Nortriptyline | 27 \pm 5.3 | 81 \pm 21 | 70 \pm 19 | 640 \pm 150 | 310 \pm 79 | 41 \pm 17 | 800 \pm 200 | 15,000 \pm 3,700 |
| Atypical antidepressants | | | | | | | | |
| Iprindole | 110 \pm 8 | 37,000 \pm 2,200 | 9,600 \pm 480 | 21,000 \pm 7,700 | 12,000 \pm 1,600 | 1,900 \pm 480 | 6,300 \pm 2,100 | 21,000 \pm 4,300 |
| Fluoxetine | 780 \pm 74 | 13,000 \pm 360 | 8,000 \pm 1,200 | 7,400 \pm 450 | 6,700 \pm 2,500 | 1,300 \pm 250 | 6,600 \pm 1,300 | 11,000 \pm 720 |
| Neuroleptics | | | | | | | | |
| Haloperidol | 1,600 \pm 730 | 110,000 \pm 28,000 | 14 \pm 2.3 | 16,000 \pm 2,000 | 960 \pm 210 | 45 \pm 2.5 | 4.2 \pm 0.80 | 28,000 \pm 940 |
| Chlorpromazine | 28 \pm 6.8 | 5,700 \pm 1,700 | 4.3 \pm 1.1 | 3,500 \pm 930 | 270 \pm 71 | 15 \pm 1.9 | 25 \pm 4.0 | 13,000 \pm 1,200 |

Note. For each drug, inhibition of 3H -labeled ligand binding was measured at four concentrations, and the median inhibitory concentration (IC_{50}) was determined by log-probit analysis. Apparent K_i values were calculated from the equation $K_i = IC_{50} / (1 + [^3H\text{-ligand}] / K_D)$, where K_D is the dissociation constant of the 3H -labeled ligand (12, 13). Each value is the mean \pm standard error of three to six experiments, each conducted in triplicate.

labeled by [^3H]spiroperidol in the corpus striatum and at β -adrenergic receptors labeled by [^3H]dihydroalprenolol ([^3H]DHA) than at any other receptor binding sites. The neuroleptics haloperidol and chlorpromazine have considerable affinities for several receptors, being similar to the more active antidepressants at α -adrenergic and serotonin-2 sites and the most potent drugs at dopamine receptors. Antidepressant potencies also vary widely at the [^3H]imipramine binding sites described by Raisman et al. (15). Blockade of any one of these receptors cannot simply account for antidepressant efficacy.

Long-term treatment with the tricyclic antidepressants amitriptyline, imipramine, and desipramine, the atypical antidepressant iprindole, and the MAO inhibitor pargyline reduces the binding of [^3H]DHA to β -adrenergic receptors; these results confirm and extend those previously reported (10, 11, 12) (Table 12-2). A substantially greater reduction of [^3H]spiroperidol binding to serotonin-2 receptors occurs after long-term treatment with antidepressants. Amitriptyline and imipramine reduce [^3H]DHA binding by 20%, but reduce [^3H]spiroperidol binding to serotonin-2 receptors by 40%. Iprindole and pargyline also reduce binding to serotonin-2 receptors more than they reduce [^3H]DHA binding. The secondary amine desipramine is the only antidepressant tested in which the decline in [^3H]DHA binding (29%) exceeds the decline in binding to serotonin-2 receptors (21%). The reductions in [^3H]DHA binding observed here are similar to those obtained by others (11, 12) at the same doses and durations of treatment. More prolonged treatment may elicit a greater reduction of both [^3H]DHA (10) and [^3H]spiroperidol (16) binding. In marked contrast, binding to β -adrenergic and serotonin receptors is unaffected by the serotonin antagonist methysergide or the neuroleptics chlorpromazine and haloperidol. Fluoxetine, a potent inhibitor of serotonin but not of norepinephrine uptake, whose antidepressant efficacy is questionable (17, 18), does not affect [^3H]DHA or [^3H]spiroperidol binding.

The binding of [^3H]serotonin to serotonin-1 receptors also declines with chronic administration of imipramine and pargyline, but not with any other antidepressant; these results resemble those of others (12, 19) and suggest that effects on serotonin-1 receptors are unrelated to antidepressant efficacy. Consistent with its labeling of both serotonin-1 and serotonin-2 receptors (14), [^3H]LSD binding is affected by long-term treatment with antidepressants in a fashion intermediate to effects on [^3H]spiroperidol and [^3H]serotonin binding. In contrast to the significant reductions of serotonin and β -adrenergic receptors, long-term drug treatment has no effect on muscarinic cholinergic receptors labeled by [^3H]QNB or α -adrenergic receptors labeled by [^3H]WB-4101. Binding to dopamine receptors labeled in the corpus striatum by [^3H]spiroperidol is increased by neuroleptic treatment (20), but is unaffected by antidepressants.

TABLE 12-2. Effect of long-term drug treatment on neurotransmitter receptor binding in rat brain

| Drug | Serotonin-2 ([³ H]spiroperidol) | Serotonin-1 + serotonin-2 ([³ H]LSD) | Serotonin-1 ([³ H]serotonin) | β-Adrenergic ([³ H]DHA) | Muscarinic cholinergic ([³ H]QNB) | α-Adrenergic ([³ H]WB-4101) | Dopamine ([³ H]spiroperidol) |
|----------------------------------|--|--|---|--|---|--|---|
| Tricyclic antidepressants | | | | | | | |
| Amitriptyline | 57±3.0* | 80±1.7* | 90±3.7 | 82±3.9† | 103±2.6 | 97±6.8 | 101±4.5 |
| Imipramine | 60±4.5* | 74±7.0† | 80±3.5† | 82±3.5† | 100±0.72 | 104±6.1 | 96±4.4 |
| Desipramine | 79±4.1* | 82±5.2† | 94±2.6 | 71±6.2† | 105±1.9 | 97±8.8 | 97±4.2 |
| Atypical antidepressants | | | | | | | |
| Iprindole | 66±4.1* | 79±6.6† | 92±6.4 | 88±1.9† | 95±2.2 | 107±3.8 | 100±2.8 |
| Fluoxetine | 87±6.3 | 92±9.7 | 98±3.8 | 97±5.8 | 97±2.1 | 102±4.3 | 99±4.0 |
| MAO inhibitor | | | | | | | |
| Pargyline | 65±5.8* | 65±0.65* | 58±2.7* | 85±3.1† | 101±0.54 | 97±2.9 | 104±1.5 |
| Serotonin antagonist | | | | | | | |
| Methysergide | 90±7.2 | 95±1.2 | 102±1.3 | 98±3.9 | 104±6.3 | 100±2.6 | 100±6.0 |
| Neuroleptics | | | | | | | |
| Chlorpromazine | 90±4.7 | 90±4.4 | 105±7.4 | 100±4.2 | 100±2.8 | 102±1.5 | 132±2.0* |
| Haloperidol | 98±6.1 | 102±4.5 | 93±3.2 | 102±5.4 | 102±0.79 | 101±2.7 | 139±6.4* |

Note. The relative amount of ³H-labeled ligand bound (saline control=100) was determined in homogenates of rat brain after 21 consecutive days of treatment with equivalent volumes of drugs or saline. Each value is the mean±standard error from three to six separate animals. The experiment was replicated twice. The concentrations of the ligands were 0.36 nM [³H]spiroperidol, 3.7 nM [³H]LSD, 2.0 nM [³H]serotonin, 0.8 nM [³H]DHA, 0.2 nM [³H]QNB, and 0.9 nM [³H]WB-4101. Specific binding was defined as the excess over blanks taken in the presence of 1 μM *d*-LSD for serotonin receptors, 1 μM (±)-propranolol for β-adrenergic receptors, 0.1 mM oxotremorine for muscarinic cholinergic receptors, 0.1 mM (–)-norepinephrine for α-adrenergic receptors, and 1 μM (+)-butaclamol for dopamine receptors. All experiments were performed in triplicate. Student's *t*-test was used for statistical analysis of the raw data before conversion to percentages.

**P*<0.01. †*P*<0.05.

Decreases in the binding of ^3H -labeled ligand may be due to changes in the number of sites or in the dissociation constant (K_D). Scatchard analysis of the binding of [^3H]spiroperidol at a wide range of concentrations indicates that the antidepressants decrease the number of serotonin-2 binding sites with no change in the affinity constant (data not shown). Similarly, a reduction in the number of [^3H]DHA binding sites with no change in K_D has been reported after antidepressant treatment (10, 11, 12). It is unlikely that changes in receptor binding are due to residual drug in the brain, since short-term injections of antidepressant fail to alter the binding to serotonin-2 or β -adrenergic receptors 24 hours after a single dose (16).

Clinically effective antidepressants have substantial affinity for a number of neurotransmitter receptor binding sites, although no correlation exists with clinical dosages. However, long-term treatment with antidepressant reduces binding to both serotonin-2 and β -adrenergic receptors, the reduction in the binding to serotonin-2 receptors being the more marked for all drugs except desipramine. Moreover, this effect appears selective, because fluoxetine, the serotonin antagonist methysergide, and the neuroleptics chlorpromazine and haloperidol fail to alter serotonin-2 or β -adrenergic binding. In neurophysiological studies (18), long-term treatment with antidepressant increases neuronal responsiveness to the inhibitory effect of serotonin. It is unclear whether this effect is related to the decrease in binding to serotonin-2 receptors. The decrease in the binding to β -adrenergic receptors is correlated with a decrease in the sensitivity of the β -adrenergic-sensitive adenylate cyclase (11).

How might these findings fit with other data on the pharmacology of depression? Depletion of monoamines by reserpine is associated with clinical depression (1). The tricyclic antidepressant blockade of uptake systems and the inhibition of MAO by pargyline both enhance amine activity. Though iprindole is considered an atypical antidepressant because of its failure to block uptake and to inhibit MAO, it presumably potentiates central biogenic amines, since it increases the central effects of amphetamine (21). Subsensitivity of serotonin-2 and β -adrenergic receptors might be secondary to chronic enhancement of amine activity. Whether the antidepressant actions stem primarily from effects on synaptic monoamines or from changes in receptor sensitivity is not clear. However, the fact that long-term treatment is required to elicit changes in receptor density whereas enhancement of monoamine action occurs immediately favors the role of receptor sensitivity in mediating antidepressant action.

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CHAPTER 13

Two Distinct Central Serotonin Receptors With Different Physiological Functions

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Serotonin (5-hydroxytryptamine) elicits both synaptic inhibition and excitation in the brain (1, 2, 3, 4) and plays a role in numerous behavioral systems. Increased concentrations of serotonin in the brain result in a behavioral hyperactivity syndrome with head twitching, resting tremor, and hyper-tonicity (5, 6). A serotonin-sensitive adenylate cyclase in brain homogenates may mediate some serotonin responses (7, 8, 9). Serotonin receptors in the brain can bind tritium-labeled lysergic acid diethylamide (LSD) (10), serotonin (11), and spiroperidol (12). Recently, we demonstrated that [^3H] serotonin and [^3H]spiroperidol bind to physically distinct populations of serotonin receptors in the brain, whereas [^3H]LSD binds to both of these sites with similar affinity (13). The receptors that bind serotonin (designated serotonin 1 receptors) are regulated by guanine nucleotides, whereas sites that bind spiroperidol (serotonin 2 receptors) are not influenced by nucleotides (14). By comparing drug affinities for serotonin binding sites with drug potencies in physiologic functions, we now provide evidence that serotonin 1 receptors

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might be related to the serotonin-sensitive adenylate cyclase, whereas the behavioral syndrome resulting from central serotonin stimulation is mediated by serotonin 2 receptors. Moreover, drug specificities of serotonin-elicited synaptic inhibition and excitation reflect serotonin 1 and serotonin 2 receptor interactions, respectively.

Behavioral hyperactivity follows central serotonin stimulation with drugs such as 5-hydroxytryptophan, tryptophan plus a monoamine oxidase inhibitor, D-LSD, and quipazine (5, 6). The syndrome includes resting tremor, hind-limb abduction, splayed hindlimbs, snake tail, side-to-side head weaving, and head twitching. The head twitch is an easily monitored and reliable measure of the presence of the syndrome. Accordingly, we evaluated the potencies of a wide range of drugs in inhibiting hydroxytryptophan-elicited head twitches in mice (Figure 13-1A, 13-1B). A number of drugs—including classical serotonin antagonists such as cyproheptadine and metergoline, neuroleptics such as spiroperidol and pipamperone, and antidepressants (15) such as mianserin and amitriptyline—are capable of preventing head twitches induced by hydroxytryptophan. Although amitriptyline and mianserin are potent inhibitors of the induced head twitches, other antidepressants, such as desipramine and iprindole, are much weaker; thus, serotonin blockade is not likely to account for the therapeutic efficacy of these latter drugs. The butyrophenone neuroleptic spiroperidol is the most potent inhibitor of hydroxytryptophan-induced head twitches; a 50% inhibitory dose (ID_{50}) of 0.18 μ mole per kilogram of body weight also inhibited apomorphine-induced stereotypy (16), a dopamine-linked behavior. Pipamperone (ID_{50} =1.73 μ mole/kg) and chlorpromazine (ID_{50} =2.41 μ mole/kg), on the other hand, are more than 100 and 8 times, respectively, more potent inhibitors of the serotonin than of the dopamine behavioral syndrome. Conversely, haloperidol (ID_{50} =4.26 μ mole/kg) is approximately 10 times more potent in blocking the dopamine than the serotonin syndrome. Thus, while neuroleptic drugs are potent antagonists of the hydroxytryptophan syndrome, these effects are not mediated through the dopamine system.

Drug affinities for serotonin 1 receptors labeled by [3H]serotonin do not correlate with head twitch blockade (Figure 13-1A). By contrast, drug potencies in blocking induced head twitches closely correlate with affinities for serotonin 2 receptors labeled by [3H]spiroperidol ($r=0.98$, $P<0.001$). Since *in vivo* blockade of head twitches correlates with *in vitro* receptor affinity, the drugs tested presumably differ little in their ability to reach target sites in the brain.

Several lines of evidence suggest that serotonin 1 receptors are associated with the serotonin-sensitive adenylate cyclase. Serotonin 1 but not serotonin 2 receptor binding is regulated by guanine nucleotides (14), a phenomenon commonly reflecting a linkage to adenylate cyclase (17).

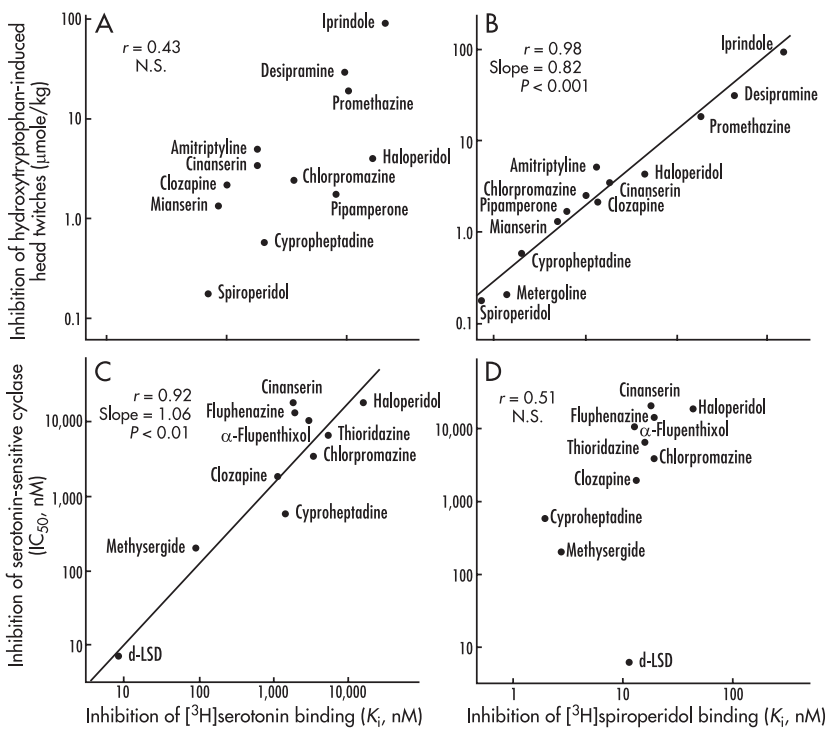


FIGURE 13-1. Comparison of drug affinities at two serotonin receptors with drug inhibition of serotonin-related behavior.

Binding assays were performed on cortical membrane homogenates prepared from freshly decapitated male Sprague-Dawley rats as described previously (13, 14). Behavioral studies were performed on mice after the injection of 300 mg of 5-hydroxytryptophan 30 minutes before a 2-minute observation period. Various drugs were injected 1 hour before the observation period (6). The number of head twitches was recorded at four or five concentrations, and ID_{50} values were calculated for log-probit analysis. Slopes of these plots were parallel for all drugs. Standard errors did not exceed 15% of mean values. Drug inhibition values (IC_{50} 's of serotonin-sensitive adenylate cyclase) were derived from previously published reports (8, 9). Depending on brain region, species, and experimental conditions, absolute potencies of drugs vary at the serotonin-sensitive cyclase (7, 8, 9). Data depicted include results from all published studies on drugs at the serotonin-sensitive adenylate cyclase (7, 8, 9). A similar high correlation with affinities for $[^3\text{H}]$ serotonin binding sites occurs regardless of the species and experimental conditions. Data were statistically compared by linear regression analysis.

TABLE 13-1. Comparison of drug potencies at serotonin receptors with physiological actions, means±standard errors of three to six experiments, each performed in triplicate

| Serotonin-related drugs | Affinity for serotonin 1 receptors: K_i versus [³ H]serotonin (nM) | Affinity for serotonin 2 receptors: K_i versus [³ H]spiroperidol (nM) | Effects on serotonin synapses | |
|-------------------------|--|---|----------------------------------|----------------------|
| | | | Inhibition | Excitation |
| Serotonin | 2.7±0.55 | 2,700±400 | Agonist (1, 2, 3, 4, 23, 24, 25) | Agonist (1, 2, 3, 4) |
| D-LSD | 9.8±1.0 | 8.9±1.7 | Agonist (3) | Antagonist (1, 2, 3) |
| Lisuride | 6.2±1.1 | 11±3.6 | Agonist (25) | |
| Metergoline | 9.9±2.1 | 2.1±0.67 | Antagonist (24) | Antagonist (4) |
| Cyproheptadine | 1,100±120 | 2.4±0.21 | No effect (4) | Antagonist (4) |
| Bromo-LSD | 100±15 | 2.5±0.41 | No effect (1) | Antagonist (1) |
| Methysergide | 150±17 | 3.1±0.91 | No effect (1, 2, 4) | Antagonist (1, 2, 4) |
| Methiothepin | 300±27 | 4.1±0.67 | No effect (4) | Antagonist (4) |
| Cinanserin | 1,800±540 | 15±3.1 | No effect (1, 4) | Antagonist (1, 4) |

Note. The influences of drugs on microiontophoretic serotonin effects are from the literature (1, 2, 3, 4, 23, 24, 25).

Though guanine nucleotides are less potent in regulating serotonin receptors than some others, the specificity of the effect is the same as at other transmitter and hormone receptors. Of course some receptors regulated by guanosine 5'-triphosphate have not been clearly linked to a cyclase (18). Kainic acid lesions of rat corpus striatum elicit parallel declines in serotonin 1 receptor binding and serotonin cyclase (19). Finally, drug potencies as inhibitors of the serotonin cyclase correlate with their affinities for serotonin 1 receptors (Figure 13-1C) ($r=0.92$, $P<0.01$). By contrast, cyclase inhibition does not correlate significantly with drug affinity for serotonin 2 receptors (Figure 13-1D). However, differences in ontogenetic development and drug sensitivity argue that serotonin 1 receptors and the serotonin-sensitive cyclase are distinct entities (20). The possible existence of multiple [^3H]serotonin-labeled receptors (21) might account for these discrepancies as may the existence of distinct serotonin-sensitive adenylate cyclase complexes with different affinities for various drugs (8, 9).

Differential neurophysiologic actions of serotonin may also reflect discrete effects at serotonin 1 or serotonin 2 receptors (Table 13-1). Synaptic excitation but not inhibition by iontophoretically applied serotonin is antagonized by drugs such as cyproheptadine, bromo-LSD, methysergide, and cinanserin, which are 40 to 400 times more potent at serotonin 2 than at serotonin 1 receptors. Lisuride, LSD, and metergoline, which influence iontophoretic serotonin inhibition, are 15 to 200 times more potent at serotonin 1 receptors than the peripheral serotonin antagonists, which fail to inhibit serotonin. Metergoline, which blocks both inhibition and excitation of serotonin, displays low nanomolar K_i values for both types of receptors. Since iontophoretic results are only qualitative, one cannot establish numerical correlations with drug affinities for receptor binding sites or behavioral potencies.

The data suggest that serotonin inhibition and excitation are mediated by serotonin 1 and serotonin 2 receptors, respectively. The excitatory actions of serotonin may reflect a facilitation of excitatory influences of other substances such as acetylcholine and glutamate (22). Since the serotonin behavioral syndrome appears to involve serotonin 2 receptors, it may reflect excitatory synaptic actions of central serotonin. The relationship between serotonin 1 receptors and both the serotonin cyclase and neural inhibition is less clear than the link of serotonin 2 sites and excitation. A major problem in evaluating serotonin 1 receptor binding, the serotonin-sensitive adenylate cyclase, and iontophoretic serotonin inhibition is the lack of specific, high-affinity antagonists. Whereas cyproheptadine has nanomolar affinity for serotonin 2 sites and a 500-fold predilection for serotonin 2 over serotonin 1 receptors, no such selective antagonist has yet been described for serotonin 1 receptors, the serotonin cyclase, or serotonin-mediated inhibition.

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Part VI

INOSITOL PHOSPHATES AND ACTIONS OF LITHIUM

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COMMENTARY

Lithium, Second Messengers, and Downstream Effects

Robert M. Post

The series of papers in this section helps make the transition from considering lithium's mechanism of action on a presynaptic basis to considering its postsynaptic mechanisms at the level of second messenger systems and their downstream effects on intracytoplasmic receptors and calcium homeostasis. As usual, Solomon Snyder's lab was at the forefront of each of the sequential series of decade-long transitions in considering the mechanisms potentially related to the action of psychotropic drugs. In the 1960s and 1970s, most drug actions were considered on the basis of presynaptic release and reuptake and related effects on specific aminergic neurotransmitter metabolism and turnover. Consideration of psychotropic medication action then shifted toward consideration of pre- and postsynaptic receptors with the discovery of the opiate receptor in Snyder's lab.

In the late 1980s and the 1990s investigators began to consider psychotropic drug action at the level of second messenger systems and downstream intracellular effects. This work, important in its own right, helped pave the way for the subsequent reconceptualization and consideration of drug actions at the level of protein kinases, transcription factors, and intranuclear effects on gene expression in the 2000s.

Lithium has neurobiological effects at multiple levels of synaptic transmission and neuronal plasticity. Not only is lithium the first and paradigmatic mood stabilizer treatment for bipolar disorder, but it is widely recognized as a first- or second-line treatment in the augmentation of anti-

depressant response in patients with unipolar major depression as well. It was originally thought that uncovering the action of lithium would lead to a better understanding of the neurochemical changes underlying manic-depressive illness and, perhaps, to novel treatment approaches. This hope has not yet been realized as discovery of the multitude and complexity of lithium's actions has outpaced the ability to assess which specific action of lithium is most crucial to its therapeutic effects in bipolar disorder.

A major contributor to this failure to dissect the mechanisms of lithium most important for the affective disorders has been the lack of suitable animal models, particularly for bipolar illness. If there were a readily available model of lithium-responsive effects on manic- and depressive-like behaviors in animals, the process of assessing which of the panoply of lithium effects is most important would be greatly simplified. Even though the hope of unlocking the mechanism of action of lithium most pertinent to its effects in bipolar illness has not yet been fulfilled, this series of groundbreaking papers on lithium's intracellular actions continues to offer a novel set of candidate mechanisms.

Lithium is best known for its acute antimanic effects, but the early work of Baastrup and Schou (1) indicated it was also effective in the prevention of both manic and depressive episodes. A body of less well verified data indicates that lithium may also have effects on the treatment of acute bipolar depression in monotherapy, in addition to its adjunctive role in the treatment of unipolar depression where it clearly augments the effects of traditional antidepressants. It is now also recognized that lithium is one of the few drugs, if not the only drug, with clear evidence of ability to prevent one of the lethal outcomes of inadequately treated affective disorders, suicide. In addition, long-term treatment with lithium normalizes the increased mortality from other medical illnesses that is associated with recurrent affective disorders. Depression is a major risk factor for the onset of many medical illnesses and/or their poor outcome. For example, the presence of depression increases the likelihood some two- to fourfold that an individual will experience a heart attack, and it also increases by some two- to fourfold the likelihood of dying of that heart attack compared with a nondepressed individual.

The broad spectrum of clinical therapeutic effects of lithium also presents problems in the ultimate assessment of which of its actions may be related to its antimanic or antidepressant effects, or in the prevention of suicide and the excess medical mortality associated with the affective disorders. Whether lithium has separate actions in its ability to prevent suicide and excess medical mortality beyond its ability to prevent manic and depressive episodes remains to be more clearly delineated.

Also complicating the process of attribution of any specific mechanism of action to a given clinical therapeutic effect is the great clinical heteroge-

neity of response to an agent such as lithium within the general population of individuals with bipolar disorder. Lithium monotherapy is the ideal treatment for only a small minority of patients with this illness. In this case, the ideal would be the prevention of manic and depressive prodromes and episodes, the long-term maintenance of that remission, and the accomplishment of this in the relative absence of side effects.

The anticonvulsants carbamazepine and valproate have emerged as alternatives or adjuncts to lithium in the treatment of acute mania and in episode prevention. Most recently, lamotrigine has been approved, not for treatment of acute manic or depressive episodes, but for their prevention. Only preliminary clinical hints are available about which patient is most likely to respond to which of these four mood stabilizers (2, 3).

The bulk of the evidence, however, suggests that predictors of good lithium response include a positive family history of bipolar I illness in first-degree relatives, a classic pattern of discrete and euphoric (as opposed to dysphoric) manic episodes, and clear-cut well intervals in between. Lithium also appears more likely to be effective in those who have the sequence of mania, depression, well interval, as opposed to those whose typical sequence is depression, mania, well interval. Also associated with a greater likelihood of response to lithium are the absence of anxiety and substance abuse comorbidities, the absence of mood-incongruent delusions, and a lesser number of affective episodes prior to beginning prophylaxis. Thus, it may emerge that specific mechanisms of lithium's actions may be related to clinical responsiveness in specific patient subtypes. Such a delineation would obviously be of great importance in helping to match individual patients with the best treatment for their subgroup or type of illness.

Therefore, it remains a possibility that the actions of lithium described in this section may be most pertinent to one subgroup of patients with the illness. If one could use a combination of clinical, neurochemical, and genetic markers, one might be able to increase the percentage of likelihood of a good response in an individual patient from the current 50% rate for many treatments to much higher levels. To date, only very preliminary data suggest that patients with the short subtype of the serotonin transporter (5HT-Tss), who typically respond poorly to traditional antidepressants, may be particularly responsive to the effects of lithium.

What may be other aspects of the potential clinical significance of this series of papers? The hypothesis of Janowsky et al. (4) suggested that the molecular pathophysiology underlying depression is that of an excess in cholinergic tone. Thus, the paper of Worley et al. (5; Chapter 14 in this volume) reveals that lithium could dampen this postulated cholinergic hyperactivity of depression via its effects on phosphoinositide second messenger systems. As discussed in the paper of Baraban et al. (6; Chapter 16), this

opens the possibility of lithium acting not only at the previously better known second messenger systems of cyclic AMP, GMP, and their associated G proteins, but also at another system that mediates the effects of a very large number of neurotransmitters.

This set of hypotheses certainly paves the way for the conceptual breakthrough that lithium may not act on one *specific* neurotransmitter system for its therapeutic effects, but rather, because of its modulatory role on second messengers, may dampen the hyperactivity or dysregulation of a number of neurotransmitters that may be importantly related to the affective disorders. This modulation of multiple systems could help solve the conundrum of how a single substance, such as the simple salt of lithium carbonate or lithium chloride, could treat and prevent both manic and depressive phases of bipolar illness with their apparent very opposite characteristics in terms of mood, motor activity, social behavior, and judgment, as well as regional brain activity, neurochemistry, and endocrinology.

Calcium is crucial not only to presynaptic neurotransmitter release, but also to postsynaptic intracellular transduction mechanisms and cellular excitability. The rapidity and amount of calcium influx through the glutamate NMDA receptor appear to be crucial in whether synapses show long-term potentiation or long-term depression. It had previously been noted that lithium had effects on calcium homeostasis, but how this occurred had not been delineated. The following papers now provide the answer.

Phosphoinositide (PI) signaling is now known to involve the splitting of phosphatidylinositol-bisphosphate (PIP_2) by phospholipase C into two components, diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG has downstream actions on the activity of protein kinase C, and IP_3 binds to its receptor on the endoplasmic reticulum and increases calcium efflux. The paper by Supattapone and colleagues (7; Chapter 15) described this first discovery and identification of the IP_3 receptor, and the paper by Ferris et al. (8; Chapter 17) uncovered how this receptor mediates calcium flux. Lithium can also affect calcium influx through the NMDA receptor and other cell surface mechanisms, but actions on the IP_3 receptor remain a key mechanism for lithium's important signaling effects.

Not only do calcium's basal levels and acute fluctuations set the tone for intracellular and synaptic excitability, but intracellular calcium oscillations are also involved in converting acute to long-term effects, and in the release of endocrine substances. In this fashion, lithium's effects on acute efflux through the IP_3 receptor or on calcium cycling could have widespread modulatory effects in multiple systems. While lithium, carbamazepine, and valproate each appear to have different effects on PI turnover, these mood stabilizers all have important effects on calcium homeostasis that could be important to their class effect as mood stabilizers.

Subsequent papers from Snyder's lab later in this volume take us to the action of psychotropic drugs at the level of the nucleus and gene transcription, as well as on energy generation via mitochondria. Lithium's effects at these levels involve its ability to increase cell survival factors, such as brain-derived neurotrophic factor and BCL-2, as well as inhibit cell death factors such as Bax and P53 (9). These proteins influence not only cellular excitability, but also cell survival or cell death. An explosive increase in intracellular calcium can lead to excitotoxicity while more subtle mechanisms lead to preprogrammed cell death or apoptosis, depending on the relative balance of cell survival and death factors. In one form of apoptosis, mitochondria release cytochrome *c*, which activates intracellular caspases, which fragment proteins and begin the process of cell membrane dissolution and cell death.

Excitotoxicity causes an inflammatory response and leaves a prominent glial scar, while the remnants of cells dying from apoptosis are quickly cleared without an obvious trace. This latter mechanism may be more pertinent to the psychiatric illnesses in which there is increasing evidence of not only chemical but also microstructural alterations in the brain, and these occur without evidence of the glial scarring that is so prominent in the brain lesions of many neurological disorders.

Thus, this series of articles indicates that Solomon Snyder has unlocked some of the secrets of lithium's effects on the modulation of intracellular transduction mechanism at the level of phosphoinositide-mediated calcium homeostasis. These calcium fluxes could be related not only to short- and long-term alterations in cellular excitability, but also to neuronal survival or disappearance via cell death. It may be only a curious coincidence that lithium, which is uniquely able to reduce the incidence of clinical suicide, now also appears involved in the inhibition of suicide at the cellular level by a variety of mechanisms.

However, it is also possible that the two phenomena are somehow related via the laws of chaos theory, reflecting self-similarity at multiple levels of temporal and spatial analysis. Snyder's lab has played a key role in the understanding of lithium's actions at multiple molecular levels. What is unique about him and his coworkers is that they have remained so closely tied to clinical medicine and the more fundamental understanding of the causes and treatments of the major psychiatric disorders. While implications for therapeutics often arise by serendipity, in the work of Solomon Snyder they seem to spring forth from a directed keener and deeper understanding of cellular neurochemistry and physiology.

The papers in this section reveal some of the many tools he and his collaborators use in this type of pioneering exploration and understanding. Mechanisms of intracellular second messenger systems are uncovered; new assays are developed; and receptors are discovered and isolated. Their

actions on calcium are explored in multiple cellular systems from hippocampal slices to reconstituted lipid vesicles, and then these mysterious data are pulled together to provide a new conceptual approach to the molecular mechanisms pertinent to the better understanding and treatment of some of the most disabling disorders from which humankind suffers. This exciting process of discovery and new synthesis has already been re-created and reiterated many, many times in this laboratory, as revealed in the other sections of this compendium, and now we all look forward in the near future to the next set of miraculous findings that surely will emanate from Solomon Snyder and his coworkers.

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CHAPTER 14

Lithium Blocks a Phosphoinositide-Mediated Cholinergic Response in Hippocampal Slices

Paul F. Worley
William A. Heller
Solomon H. Snyder
Jay M. Baraban

The discovery that the phosphoinositide (PI) system is a prominent second messenger for neurotransmitters has led to the suggestion that the influence of lithium on inositol phosphate metabolism accounts for its psychotropic actions (1). According to this hypothesis, the blockade by lithium of the enzymatic hydrolysis of inositol monophosphate (2) alters PI signal transduction by limiting the regeneration of inositol, an essential precursor in PI synthesis. At present, however, there is little evidence that lithium affects PI-mediated neurotransmitter responses.

Muscarinic activation of the PI system in hippocampal slices, either by electrical stimulation of intrinsic cholinergic fibers or by bath application of muscarinic agonists, blocks the inhibitory action of adenosine on synaptic transmission (3, 4). The action of adenosine is blocked by oxotremorine-M,

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which strongly stimulates the PI cycle in the hippocampus, but not by oxotremorine, a weak partial muscarinic agonist of PI turnover (5). Instead, oxotremorine antagonizes oxotremorine-M stimulation of PI turnover (5) and blockade of adenosine (3). Cholinergic inhibition of adenosine appears to involve protein kinase C (PKC) activation, since phorbol esters, which stimulate PKC activity, reproduce the effect of muscarinic agonists (3, 6). We now show that therapeutic concentrations of lithium selectively impair muscarinic cholinergic responses in the hippocampus that are mediated by the PI cycle.

Since lithium's predicted effects on PI-mediated neurotransmission should depend on the level of stimulation of PI turnover, we assessed the effects of carbachol, a muscarinic agonist, on adenosine responses during prolonged application. Cholinergic stimulation of the PI cycle monitored biochemically in the hippocampus persists for more than 1 hour (7). Similarly, we found that the adenosine responses were blocked throughout 1 hour of carbachol application (Figure 14-1). The action of carbachol is reversible, since adenosine's potency is restored when carbachol is washed away (3). Furthermore, reapplication of carbachol restored the blockade of adenosine during a second hour of incubation (Figure 14-1). To test the actions of lithium on this PI-mediated response, we ensured that carbachol blocked adenosine for 1 hour, and then we added lithium to the buffer 1 hour before a second challenge with carbachol. Under these conditions, lithium impaired the ability of carbachol to block adenosine (Figure 14-1). Similar results were obtained when oxotremorine-M was used instead of carbachol.

The effect of lithium was concentration-dependent, with a partial effect at 0.5 mM lithium and near maximal effects at 1 mM and 2 mM lithium (Figure 14-2). The inhibition of carbachol by lithium was also time dependent, beginning after 10 to 15 minutes of incubation, with submaximal and maximal effects apparent at 30 and 60 minutes, respectively. To ascertain the ionic selectivity of lithium, we examined the effect of rubidium, which does not inhibit inositol monophosphate hydrolysis (2). At concentrations of rubidium up to 2 mM, no reversal of the carbachol effect was observed.

Because the actions of lithium are reversible, a general toxic effect on the slices is unlikely. After exposure to lithium and carbachol under standard conditions, the slices were supervised for 60 minutes with control saline that did not contain lithium or carbachol. At the end of this period, the ability of carbachol to reverse adenosine's effect was restored and persisted throughout 60 minutes of application of carbachol, essentially as observed in fresh slices ($n=4$).

Because cholinergic reversal of the effect of adenosine appears to involve PKC, we examined the influence of lithium on the effects of phorbol 12,13-diacetate (PDA) on adenosine. At a time when the blockade of adenosine by

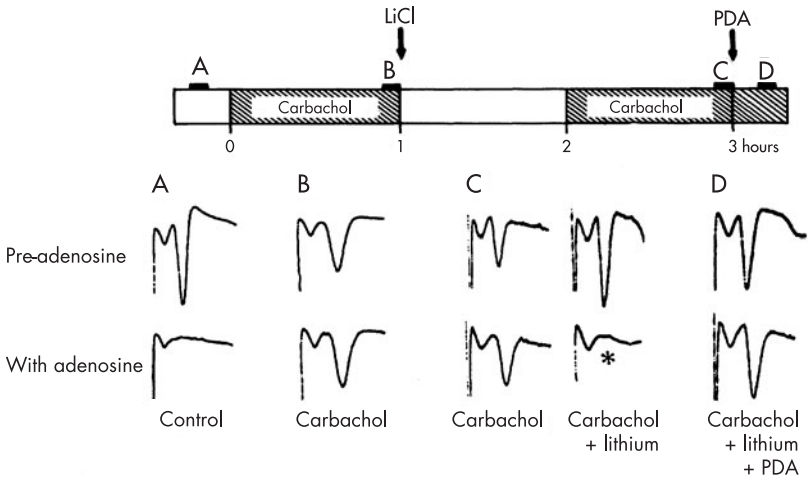


FIGURE 14-1. Lithium blocks the PI-mediated response to carbachol.

The protocol used to monitor carbachol's block of adenosine is shown. The sequence and duration of drug applications are indicated on the bar above tracings of the CA1 population spike (PS) elicited by stimulation of Schaffer collaterals (0.1 Hz), for which standard recording conditions were used (3). Letters above the bar indicate times at which tracings were taken. Traces in the top row were made before application of adenosine, those in the bottom row during maximal response to adenosine at the end of a 5-minute application. (A) Application of adenosine (80 μ M) reversibly suppressed the PS. (B) Carbachol (50 μ M), applied for 1 hour, blocked adenosine's inhibitory action. Slices were then washed for 1 hour with control saline or saline containing lithium chloride (1 mM). (C) After a 1-hour reapplication of carbachol, the PS monitored in control saline remained resistant to adenosine, whereas the PS recorded in the presence of lithium chloride was reversibly suppressed by adenosine (*). (D) Subsequent addition of 2 μ M PDA to lithium-containing saline restored blockade of adenosine. Data shown are from one pair of representative experiments. Group data are presented in Figure 14-2. As reported previously (3), application of carbachol transiently suppresses the PS, which returns to approximately two-thirds of initial amplitude as shown by the smaller PS in the top row of (B) and first column of (C) as compared to (A). Tracings shown in the second column of (C) and in (D) are taken from another slice, which was treated with lithium chloride (1 mM) after the first hour of carbachol administration.

carbachol was abolished by lithium, PDA still reversed the effect of adenosine (Figure 14-1), an indication that lithium acts in the PI cascade before PKC activation.

The cholinergic blockade by lithium appears to involve the PI cycle. In hippocampal slices, lithium elevates concentrations of inositol 1-phosphate in the presence of muscarinic agonists (7, 8). Moreover, lithium treatment

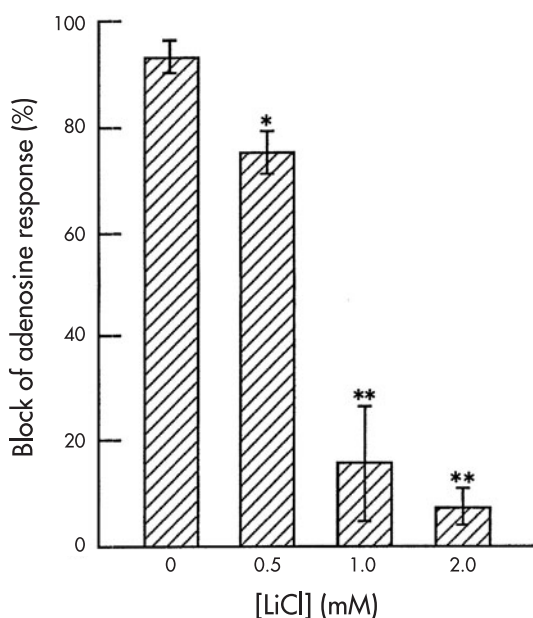


FIGURE 14-2. Concentration dependence of lithium's reversal of the carbachol block of adenosine.

The efficacy of the block of adenosine by carbachol was assessed after the second hour of carbachol application as described in Figure 14-1 by comparing the PS amplitude before and after application of adenosine (80 μ M). Adenosine completely suppressed the PS in the absence of carbachol but was minimally effective in the presence of carbachol (0 mM LiCl). Administration of 1 mM or 2 mM LiCl along with carbachol greatly reduced carbachol's block of adenosine. Data are the mean values of the number of determinations with bars indicating the standard error of the mean. Statistical analysis was performed by Student's *t*-test. Significant differences shown are as follows: * $P < 0.03$; ** $P < 0.003$ with Bonferroni correction for multiple comparisons. Number of determinations were as follows: for 0 mM LiCl, $n = 13$; for 0.5 M LiCl, $n = 3$; for 1 mM LiCl, $n = 6$; and for 2 mM LiCl, $n = 11$.

of rats *in vivo* reduces muscarinic stimulation of PI turnover in brain slices, which has been measured biochemically (9). Rubidium, which does not inhibit inositol phosphatases, does not reproduce the effects of lithium. The requirement for prolonged stimulation with carbachol to demonstrate the lithium block fits well with the time required for the buildup of inositol phosphates and the presumed depletion of substrates for the PI cycle.

How might lithium block PI-mediated cholinergic responses in hippocampal slices? The cholinergic response we have observed appears to involve PKC since it is reproduced by phorbol esters. Since the response to

phorbol esters in lithium-treated slices is normal, activation of PKC is apparently impaired by lithium treatment. This finding fits well with the inhibition of muscarinic-stimulated PI turnover by lithium (9).

We showed earlier that therapeutic concentrations of lithium retard the relaxation of smooth muscle elicited either by cholinergic or histamine stimulation acting through the PI cycle (10). This result may also reflect a diminished activation of PKC, since under the experimental conditions used phorbol esters relax smooth muscle (11).

Our study demonstrates that alterations in PI-associated central neurotransmission occur at therapeutic concentrations of lithium. These short-term effects of lithium might reflect changes leading to the delayed therapeutic actions of lithium in affective disorders. Numerous neurotransmitters act through the PI system. The dampening action of lithium on PI-associated neurotransmission could prevent excessive excursions of individual neurotransmitter systems from basal activity, with different PI-linked transmitters separately involved in manic and depressive episodes. This model would explain the normalizing action of lithium in providing symptomatic relief and serving as prophylaxis against episodes of both mania and depression (12).

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CHAPTER 15

Solubilization, Purification, and Characterization of an Inositol Trisphosphate Receptor

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The phosphoinositide second messenger system provides a transduction mechanism for a multiplicity of hormones, neurotransmitters, and growth factors (1, 2, 3, 4, 5). In the phosphoinositide cycle, phosphatidylinositol 4,5-bisphosphate is converted to diacylglycerol and inositol 1,4,5-trisphosphate (InsP_3) following receptor activation (1, 6). InsP_3 is thought to bind to an intracellular membrane site to release calcium (1, 7). Direct evidence for InsP_3 receptor proteins has been obtained through the demonstration of saturable, high affinity binding sites for radiolabeled InsP_3 in permeabilized hepatocytes and neutrophils as well as membrane fractions from the anterior pituitary, liver, and adrenal cortex (8, 9, 10, 11). We have demonstrated high affinity saturable binding of $[^3\text{H}]\text{InsP}_3$ to membrane fractions from the rat cerebellum (12, 13). In both brain tissue and peripheral tissues the receptor demonstrates high selectivity for InsP_3 in contrast to other inositol phos-

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phates, paralleling the relative ability of these substances to release calcium. InsP_3 binding levels in the cerebellum are several hundred times greater than in peripheral tissues, permitting a detailed characterization of the receptor (13). In the present study we have solubilized the $[\text{}^3\text{H}]\text{InsP}_3$ receptor from rat cerebellar membranes, purified the protein to apparent homogeneity, and characterized some of its properties.

Experimental Procedures

Materials

$[\text{}^3\text{H}]\text{Ins-1,4,5-P}_3$ (3.6 Ci/mmol) was obtained from Du Pont-New England Nuclear. InsP_3 was obtained from Amersham Corp. or Sigma, InsP_1 was obtained from Amersham Corp. Inositol-1,3,4,5- P_4 was generously provided by Drs. R. Irvine and M. Berridge (Cambridge, UK). Phospholipase A_2 from bee venom, type I phospholipase C from *Clostridium perfringens*, and type I phospholipase D from cabbage were obtained from Sigma. Lectins were purchased from Boehringer Mannheim. Proteases were purchased from Worthington. All other reagents were obtained from Bio-Rad or Sigma.

$[\text{}^3\text{H}]\text{InsP}_3$ binding in crude membranes. $[\text{}^3\text{H}]\text{InsP}_3$ binding to rat cerebellar membranes was assayed as described previously (12, 13). For routine studies, cerebella from adult male Sprague-Dawley rats (200–300 g) were homogenized (Polytron setting 9, 10 s) in 50 volumes of ice-cold buffer A (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM β -mercaptoethanol), pelleted by centrifugation (35,000g for 10 min), and resuspended in 50 volumes of buffer A. Binding assays contained ≈ 10 mg wet weight of tissue (≈ 0.6 mg of protein) and 2.5 nM $[\text{}^3\text{H}]\text{InsP}_3$ in a total volume of 1 mL of buffer A. Assays were incubated 10 min at 4°C followed by centrifugation at 10,000g for 5 min (Beckman Microfuge 12) and aspiration of the supernatant. Pellets were suspended in 5 mL of Formula 963 liquid scintillation mixture (Du Pont-New England Nuclear) and their radioactivity determined. Nonspecific binding was determined in the presence of 1 μM InsP_3 . The stability of $[\text{}^3\text{H}]\text{InsP}_3$ was assessed for each incubation condition using open-column anion-exchange chromatography (Dowex AG 1-X8) (14, 15). In certain experiments, InsP_3 phosphatase activity was assayed by chromatographically monitoring the conversion of $[\text{}^3\text{H}]\text{InsP}_3$ to $[\text{}^3\text{H}]\text{InsP}_2$ (15).

$[\text{}^3\text{H}]\text{InsP}_3$ binding in detergent-solubilized samples. $[\text{}^3\text{H}]\text{InsP}_3$ binding to soluble fractions was assayed by the method of spun-column chromatography. Tissue samples solubilized in buffer A containing detergent were incubated with 12.5 nM $[\text{}^3\text{H}]\text{InsP}_3$ in a total volume of 200 μL for 10 min at 4°C, then loaded onto 1-mL columns packed with 70 g/liter Bio-Gel P-10 resin

and centrifuged at 1,000g for 6 min. Five mL of liquid scintillation mixture were added to the void volume of these columns and radioactivity was determined. [³H]InsP₃ binding was linear with tissue concentration from 0 to 2 mg/mL in crude detergent-solubilized membranes.

Other Methods

Protein was determined by the method of Bradford (16). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (17). Gels were stained either by Coomassie Brilliant Blue or silver (18). InsP₃ 5-phosphatase activity was assayed by a standard protocol (15).

Results

Effects of Reagents and Enzymes on [³H]InsP₃ Binding to Rat Cerebellar Membranes

Prior to attempting solubilization of the receptor, we evaluated the influence of various reagents and enzymes (Table 15-1). [³H]InsP₃ binding to intact cerebellar membranes is abolished by 1 mM para-chloromercuribenzoate (pCMBS), an effect which is prevented by pretreatment with 1 mM β-mercaptoethanol, suggesting a role for sulfhydryl groups in receptor binding. Dithiothreitol (1 mM) or 1 mM β-mercaptoethanol alone each provide limited stimulation of binding.

[³H]InsP₃ binding is abolished by α-chymotrypsin and trypsin, consistent with the receptor being a protein. Extraction with high salt does not alter [³H]InsP₃ binding to membranes, suggesting that the receptor is an integral membrane protein. Denaturing treatments such as 1 M urea or heating at 60° C for 1 h also eliminate binding. [³H]InsP₃ binding is unaffected by bacterial preparations of phospholipase C, D, or A₂. Soybean or lima bean lectins diminish binding somewhat suggesting that the receptor might be a glycoprotein. Additional support for this notion comes from the adherence of purified receptor to concanavalin A columns (see below).

Solubilization of [³H]InsP₃ Binding Sites by Detergents

We treated membrane fractions with detergent solutions for 30 min at 4°C and examined [³H]InsP₃ binding in the supernatant and particulate fractions. In the absence of detergent, all [³H]InsP₃ binding is recovered in the membrane fraction with no detectable binding in the supernatant. [³H]InsP₃ binding appears to be inactivated by sodium dodecyl sulfate, cholate, or deoxycholate. Some detergents, such as digitonin and Tween, provide very little solubilization. The most extensive solubilization occurs with 1% Triton X-100, which provides virtually complete solubilization of receptor binding

TABLE 15-1. Effects of enzymes and reagents on [³H]InsP₃ binding

| Binding treatment | [³ H]InsP ₃ binding |
|--|--|
| | % control |
| pCMBS (1 mM) | 0 |
| pCMBS (1 mM)+β-mercaptoethanol (2 mM) | 110 |
| β-Mercaptoethanol (1 mM) | 120 |
| Dithiothreitol (1 mM) | 130 |
| α-Chymotrypsin (1 unit, 15 min, 25°C) | 0 |
| Trypsin (0.1 unit, 5 min, 25°C) | 100 |
| Trypsin (0.1 unit, 15 min, 25°C) | 40 |
| Trypsin (0.1 unit, 45 min, 25°C) | 0 |
| Heat (1 h, 60°C) | 0 |
| Urea (1 M) | 20 |
| Soybean lectin (0.3 mg/mL) | 50 |
| Lima bean lectin (0.6 mg/mL) | 70 |
| Phospholipase C (1.25 units, 10 min, 25°C) | 100 |
| Phospholipase D (1.25 units, 10 min, 25°C) | 100 |
| Phospholipase A ₂ (1.5 units, 10 min, 25°C) | 100 |
| NaCl (1 M) | 100 |
| KCl (1 M) | 100 |

Note. Rat cerebella were homogenized in ice-cold 50 mM Tris-HCl, pH 8.3. The homogenate was then centrifuged at 20,000g for 15 min at 4°C and the supernatant decanted. Membranes were resuspended in 50 mM Tris, pH 8.3, and aliquots containing 0.5 mg of protein were treated with various reagents and enzymes as indicated. After appropriate time intervals, samples were centrifuged at 10,000g in a Beckman Microfuge 12 for 5 min at 4°C, the supernatant was aspirated, and membranes were assayed for [³H]InsP₃ binding (see "Experimental Procedures"). The experiment was conducted twice in triplicate and data presented are mean values which varied less than 20%.

and full recovery of the binding in original membrane preparations. Three percent Triton appears to degrade binding somewhat with a lower recovery in the supernatant fraction. At 0.1% Triton X-100, only one-fourth as much [³H]InsP₃ binding is solubilized as with 1% detergent. The solubilization by Triton is dependent upon tissue concentration with optimal solubilization and recovery at 50 mg/mL wet weight, while at 500 mg/mL wet weight the extent of solubilization is reduced by 90%.

To determine if the binding site apparently solubilized by 1% Triton X-100 is contained in slowly sedimenting membranes, we centrifuged a solubilized preparation at 120,000g for 2 h. Following this treatment all the binding is recovered in the soluble fraction with none remaining in the particulate material.

TABLE 15-2. [³H]InsP₃ binding parameters

| Preparation | K _D | B _{max} (protein) | Hill coefficient |
|------------------------------|----------------|----------------------------|------------------|
| | nM | pmol/mg | |
| Crude membranes | 80 | 5 | 1.0 |
| Triton-solubilized membranes | 85 | 6.25 | |
| Purified receptor | 100 | 4,500 | |

Note. Saturation binding experiments were performed as described under “Experimental Procedures” utilizing centrifugation assays for crude membrane samples and spun-column assays for solubilized and purified samples. Displacement of [³H]InsP₃ binding by increasing concentrations of nonradioactive InsP₃ allowed calculation of K_D and B_{max} by Scatchard transformation. Typically, 10% of the total [³H]InsP₃ was bound. This experiment was performed twice in triplicate and data presented are mean values which varied less than 20%.

The binding site solubilized with 1% Triton X-100 displays the same properties as the binding site in membrane fractions. Scatchard analysis indicates a single binding site with a K_D of about 80 nM in both membrane and solubilized preparations (Table 15-2; Figure 15-1). The inositol phosphate specificity of binding is also the same in solubilized membrane fractions with InsP₃ being the only inositol phosphate displaying potent inhibition of binding (Table 15-3).

In most studies of [³H]InsP₃ binding to the purified receptor, 1% Triton X-100 is employed. However, even when the detergent concentration is reduced to 0.02%, [³H]InsP₃ binding to solubilized fractions is retained.

Purification of [³H]InsP₃ Binding Site

We initially homogenized cerebellar membranes in a buffer containing 1 mM EDTA and 1 M NaCl. The membranes were then subjected to three additional washes with a buffer containing 1 mM EDTA without salt. This washing procedure provides essentially 100% yield of [³H]InsP₃ binding in the membranes with none apparent in the soluble fraction. Solubilization of washed membranes with 1% Triton X-100 provides complete recovery of ligand binding in the soluble fraction (Table 15-4).

The [³H]InsP₃ binding site adheres to DEAE-cellulose and is retained on the column after a wash with 0.1 M NaCl. Binding activity is eluted with 0.175 M NaCl providing a 5-fold purification with high levels of recovery.

Earlier we showed that heparin potently inhibits [³H]InsP₃ binding to membrane fractions (13). Accordingly, we adsorbed the eluate from the DEAE-cellulose column to a heparin-agarose column. The receptor adheres to the column following a wash with 0.25 M NaCl but is eluted with 0.5 M

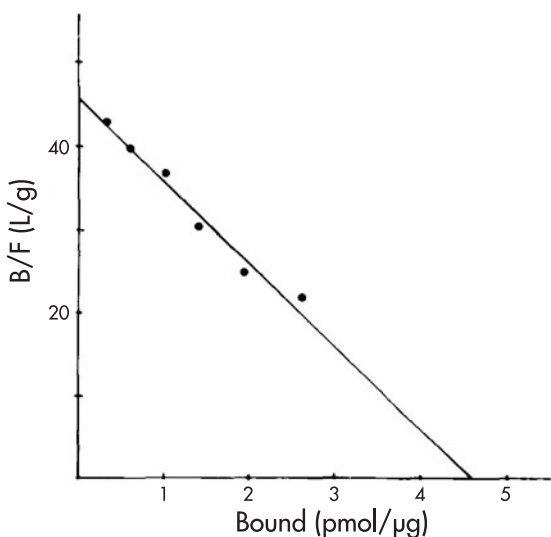


FIGURE 15-1. Scatchard analysis of $[^3\text{H}]\text{InsP}_3$ binding to purified InsP_3 receptor.

Saturation of $[^3\text{H}]\text{InsP}_3$ binding was examined with purified InsP_3 receptor by utilizing spun-column assays. Assays were conducted with $0.1\ \mu\text{g}$ of pure InsP_3 receptor, $12.5\ \text{nM}$ $[^3\text{H}]\text{InsP}_3$, and various concentrations of nonradioactive InsP_3 . Less than 10% of the total $[^3\text{H}]\text{InsP}_3$ was bound in all determinations.

NaCl providing a 160-fold purification with an 80% yield. The receptor binds to concanavalin A-Sepharose and can be eluted with $1\ \text{M}$ α -methylmannopyranoside, indicating that it is a glycoprotein.

Further fractionation of the receptor on a gel filtration column (Sephacryl S-400) affords no further purification. The binding site elutes somewhat earlier than thyroglobulin indicating an approximate Stokes radius of $10\ \text{nm}$. Electron microscopic examination of the purified protein indicates that it is globular in nature. For a globular protein, this Stokes' radius suggests a native molecular weight of approximately $1,000,000$. This value may be an overestimate since Triton X-100 bound to the protein would tend to increase its apparent Stokes' radius.

We examined the binding protein purified through the heparin-agarose step by SDS-PAGE analysis (Figure 15-2). A protein band of $260,000$ is the only one apparent on the gel. This protein band comigrates with $[^3\text{H}]\text{InsP}_3$ binding activity in fractions eluted from the Sephacryl S-400 column (data not shown).

Based on the B_{max} in crude cerebellar membranes of $5\ \text{pmol/mg}$ protein and the purification of 900-fold, we calculate a molecular weight of $230,000$ for a receptor which binds InsP_3 stoichiometrically. This value corresponds

TABLE 15-3. Inhibition of [³H]InsP₃ binding by various inositol derivatives, heparin, and calcium

| | [³ H]InsP ₃ binding | | |
|--|--|-----------------------|----------|
| | Crude membrane | Detergent solubilized | Purified |
| | % of control | | |
| Control | 100 | 100 | 100 |
| 50 nM Ins-1,4,5-P ₃ | 86 | 63 | 64 |
| 100 nM Ins-1,4,5-P ₃ | 59 | 56 | 52 |
| 1 μM Ins-1,4,5-P ₃ | 14 | 26 | 10 |
| 1 μM Ins-1,3,4,5-P ₃ | 96 | 95 | 98 |
| 10 μM InsP ₆ | 85 | 91 | |
| 10 μM Ins-1-P ₁ | 92 | 92 | 95 |
| 10 μM Ins(SO ₄) ₆ | 84 | 116 | 103 |
| 10 μg/mL heparin | 54 | 59 | 40 |
| 200 nM Ca ²⁺ | 98 | 92 | 100 |
| 10 μM Ca ²⁺ | 18 | 10 | |
| 500 μM Ca ²⁺ | 12 | 0 | 102 |
| 1.5 mM Ca ²⁺ | | | 102 |

Note. [³H]InsP₃ binding to crude membranes was assayed as described under “Experimental Procedures.” [³H]InsP₃ binding to detergent-solubilized and purified samples was assayed by the spun-column assay (see “Experimental Procedures”). Calcium was buffered by EDTA. Data are means of four determinations which varied less than 20%.

well with the value from SDS-PAGE analysis. Stoichiometric binding of InsP₃ is supported by our earlier observation of a Hill coefficient of one for [³H]InsP₃ binding to cerebellar membranes (13).

Properties of the Purified InsP₃ Receptor Protein

The binding characteristics of the purified receptor are closely similar to those of the initial detergent-solubilized fractions and the membrane-bound receptors. Scatchard analysis reveals a single binding component with a K_D of about 100 nM, similar to the membrane-bound and detergent-solubilized preparations (Table 15-3). The relative potencies of inositol phosphates are the same in competing for [³H]InsP₃ binding to purified, detergent-solubilized and membrane-bound fractions (Table 15-3). InsP₃ provides 50% inhibition at concentrations slightly below 100 nM and 75–90% inhibition at 1 μM. By contrast, 1 μM InsP₄, 10 μM InsP₆, 10 μM InsP₁, and 10 μM inositol hexasulfate all do not alter [³H]InsP₃ binding to any of the receptor preparations. Heparin inhibits [³H]InsP₃ binding to a similar extent in the three receptor preparations.

TABLE 15-4. Purification of the [^3H]InsP $_3$ receptor from rat cerebellar membranes

| Fraction | Protein | [^3H]InsP $_3$ binding | Specific activity | Purification | Yield |
|--------------------------------|---------|-----------------------------------|-------------------|--------------|-------|
| | mg | pmol | pmol/mg | -fold | % |
| Cerebellar membranes | 30 | 8.8 | 0.29 | 1 | 100 |
| 1% Triton X-100 solubilization | 28 | 10 | 0.37 | 1.25 | 116 |
| DEAE-cellulose | 4 | 7.4 | 1.8 | 6.25 | 83 |
| Heparin-agarose | 0.020 | 5.9 | 290 | 1,000 | 66 |
| Concanavalin A | 0.010 | 3.0 | 300 | 1,030 | 34 |
| Sephacryl S-400 | 0.005 | 1.3 | 270 | 900 | 15 |

Note. Sixteen rat cerebella were homogenized in 20 volumes of ice-cold buffer A containing 1 M NaCl and the homogenate was centrifuged at 20,000g for 15 min. The supernatant was decanted and the pellet was homogenized in 20 volumes of ice-cold buffer A and centrifuged at 20,000g for 15 min. This extraction in buffer A was repeated two more times. The final pellet is referred to as “cerebellar membranes.” These cerebellar membranes were resuspended in 50 mg/mL wet weight with buffer A containing 1% Triton X-100. This mixture was incubated for 30 min on ice and then centrifuged at 120,000g for 2 h. The supernatant was removed and its salt concentration was adjusted to 0.1 M NaCl by addition of a concentrated salt solution. This solution was then passed over a 70-mL DEAE-cellulose column equilibrated in buffer A with 1% Triton and 0.1 M NaCl. The column was washed with 200 mL of column buffer and eluted by increasing the salt concentration to 0.175 M NaCl. During elution, the outflow of the DEAE-cellulose column was hooked in series to a 3-mL heparin-agarose column. Following elution of the DEAE-cellulose column, the two columns were disconnected and the heparin-agarose column was washed with 20 mL of buffer A 0.1% Triton and 0.25 M NaCl and eluted with 3 mL of 50 mM Tris, pH 7.7, 1 mM β -mercaptoethanol, 0.1% Triton, 0.5 M NaCl. The eluate was applied to a 0.5 \times 1-cm column of concanavalin A-Sepharose, washed with 10 mL of 50 mM Tris, pH 7.7, 1 mM β -mercaptoethanol, 0.1% Triton, 0.1 M NaCl, and then eluted in 3 mL of the same buffer with 1 M α -methylmannopyranoside. The eluate was applied to a 1.5 \times 50-cm column of Sephacryl S-400 equilibrated in buffer A with 0.1% Triton. The gel filtration column was run at 0.1 mL/min and 3-mL fractions were collected. All operations were carried out at 4°C. Binding assays were performed as described under “Experimental Procedures” utilizing 12.5 nM [^3H]InsP $_3$. This purification scheme has been replicated 5 times. Data presented are from a typical experiment.

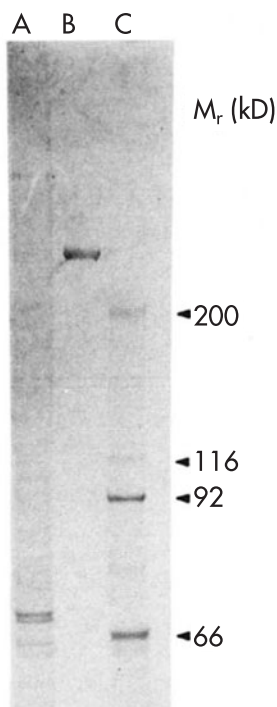


FIGURE 15-2. SDS-PAGE analysis of purified InsP_3 receptor.

A 3%–17% exponential gradient polyacrylamide gel stained with Coomassie Blue. (A) 40 μg of crude Triton X-100-solubilized cerebellar membranes. (B) 2 μg of purified InsP_3 receptor. (C) Molecular weight markers: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (92 kDa), and bovine serum albumin (66 kDa). Silver staining displayed no additional protein bands.

One striking difference between the purified and unpurified receptor involves the effect of calcium. While 500 μM calcium reversibly inhibits $[^3\text{H}]\text{InsP}_3$ binding in the particulate and detergent-solubilized receptors, it has no effect on binding to the purified receptor protein.

The dependence upon pH is the same in purified, detergent-solubilized, and particulate receptors. As reported previously (13), $[^3\text{H}]\text{InsP}_3$ binding increases 6-fold between pH 7.0 and 8.5 and remains constant between pH 8.5 and 9.5 (data not shown).

We were concerned about the possibility that InsP_3 binds to the InsP_3 5-phosphatase. This appears to be unlikely for the following reasons: 1) the affinity of InsP_3 for the receptor binding sites is nearly 1,000 times greater than the half-saturating concentration of InsP_3 for the phosphatase (11, 12, 13); 2) heparin does not inhibit the phosphatase but does displace InsP_3

binding (13); 3) the molecular weight for the [^3H]InsP₃ binding site is markedly different from that of the InsP₃ phosphatase (15); 4) the InsP₃ phosphatase is largely soluble, while the InsP₃ receptor binding site is entirely particulate. To further examine this question, we fractionated detergent-solubilized crude cerebellar membranes on a heparin-Sepharose column. Eighty percent of the phosphatase activity is eluted at 0.1 M NaCl and another 20% at 0.25 M NaCl. No [^3H]InsP₃ binding is eluted at either of these concentrations, while increasing the NaCl concentrations to 0.5 M elutes all [^3H]InsP₃ binding. Taken together with the other results, we conclude that the [^3H]InsP₃ receptor binding site is distinct from InsP₃ phosphatase.

Tissue Factors Mediating the Sensitivity of [^3H]InsP₃ Binding to Calcium

Calcium potentially inhibits [^3H]InsP₃ binding to membrane-bound and detergent-solubilized fractions in a reversible fashion. Inhibition by calcium can be reversed either by washing membranes with 1 mM EDTA or by adding 1 mM EDTA after treatment with 500 μM CaCl₂. The reversibility of inhibition by calcium rules out the possibility that calcium merely activates a protease that degrades ligand binding activity.

Following purification of the InsP₃ receptor protein, calcium (50–500 μM) fails to inhibit [^3H]InsP₃ binding (Table 15–5). We wondered whether purification of the receptor removes a tissue component which confers sensitivity of the InsP₃ receptor to inhibition by calcium. Accordingly, we examined various preparations for their ability to restore sensitivity of the purified receptor to calcium. Calmodulin is well known to bind calcium potently, but neither 2.8 nor 28 $\mu\text{g/mL}$ calmodulin in the presence or absence of calcium affects [^3H]InsP₃ binding to purified receptors. Furthermore, the purified receptor fails to adhere to a calmodulin-agarose column either in the presence or absence of 1 mM CaCl₂.

Soluble supernatant fractions of the cerebellum also fail to confer calcium sensitivity to InsP₃ binding. By contrast, detergent-solubilized membrane fractions confer calcium sensitivity on InsP₃ binding in a concentration-dependent fashion. The solubilized membrane fractions are capable of restoring completely the sensitivity of InsP₃ binding to inhibition by calcium (Table 15–5). This inhibition can be completely reversed by subsequent addition of 1 mM EDTA. The potency of calcium in inhibiting [^3H]InsP₃ binding to purified receptors in the presence of solubilized cerebellar extract is the same as in intact membrane receptor preparations (data not shown). The factor which confers calcium sensitivity appears to be a protein as its activity is destroyed by treatment with trypsin or heating (Table 15–5). Membranes from heart, liver, testes, and kidney solubilized with 1% Triton do not reconstitute calcium sensitivity.

TABLE 15-5. Inhibition of [³H]InsP₃ binding to purified InsP₃ receptor preparations by calcium

| Addition | [³ H]InsP ₃ binding (cpm) | |
|--|--|------------|
| | 500 μM calcium | No calcium |
| Purified InsP ₃ receptor alone | 1,500 | 1,480 |
| +Calmodulin 28 μg/mL | 1,480 | 1,470 |
| 2.8 μg/mL | 1,490 | 1,480 |
| +Cerebellar soluble fraction (10 mg/mL) | 1,000 | 1,000 |
| +Heparin-absorbed solubilized cerebellar membranes (1 mg/mL) | 20 | 1,500 |
| +Heat-treated membranes (1 mg/mL) | 1,000 | 1,490 |
| +Trypsin-treated membranes (1 mg/mL) | 1,500 | 1,470 |

Note. The InsP₃ receptor was purified as described in the legend to Table 15-4. [³H]InsP₃ binding was assayed by the spun-column technique as described under "Experimental Procedures." Data are given for 1.5 mM added calcium (500 μM final concentration in the presence of 1 mM EDTA) although similar results were obtained for a range of calcium concentrations (1 μM to 2 mM final concentrations). Cerebellar membranes were solubilized with 1% Triton in buffer A for 30 min at 4°C. The solubilized samples were then centrifuged at 20,000g for 1 h at 4°C and the supernatant passed over a 3-mL heparin-agarose column to remove the InsP₃ receptor. Heat treatment of membranes occurred at 60°C for 1 h. For trypsin treatment, 1 unit of enzyme was incubated for 15 min at 25°C at which time 5 units of soybean trypsin inhibitor were added to terminate proteolysis. This set of experiments has been performed three times with the same results.

Discussion

In this study we have purified the InsP₃ receptor-binding protein to apparent homogeneity from rat cerebellum. The purified receptor protein appears as a band of 260,000 on SDS-PAGE. The potent and reversible inhibition by calcium of InsP₃ binding to the receptor in its membrane-bound state or after initial detergent solubilization fits with a role for the InsP₃ receptor in mobilizing calcium from intracellular stores. Calcium does not inhibit InsP₃ binding to the purified receptor protein, but addition of a detergent-solubilized membrane preparation restores the inhibitory effect of calcium, suggesting that in intact membranes the InsP₃ receptor is closely associated with a calcium-binding protein which regulates the InsP₃ receptor. In peripheral tissues [³H]InsP₃ binding to membrane fractions is not inhibited by calcium (8, 9, 10, 11), implying that the postulated calcium-binding protein in cerebellar membranes does not occur in peripheral tissues or is not linked in these tissues to InsP₃ receptors. Since the InsP₃-binding protein of periph-

eral tissues has not been solubilized and purified, we do not know whether it represents the same protein as the receptor we have purified from the brain.

Three groups (19, 20, 21) have previously described cerebellum-specific proteins resembling the IP_3 receptor in molecular weight and tissue concentration. One of these proteins, PCPP 260 (19), is a substrate for protein kinase A (cAMP-dependent protein kinase). In preliminary work, we have observed phosphorylation of the $InsP_3$ receptor by protein kinase A, but not protein kinase C or Ca^{2+} /calmodulin-dependent protein kinase. Phosphorylation of the $InsP_3$ receptor by protein kinase A might provide a mechanism for "cross-talk" between the cAMP and phosphoinositide second messenger systems.

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CHAPTER 16

Second Messenger Systems and Psychoactive Drug Action

Focus on the Phosphoinositide System and Lithium

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Most of the major drugs used in psychiatry were introduced some 3 decades ago. In intervening years much progress has been made in understanding their mode of action in eliciting both therapeutic and side effects. Essentially all of the known actions of psychotropic drugs involve a handful of neurotransmitter systems. Specific sites of action vary among the different drugs: influences are exerted on metabolic enzymes, storage processes in synaptic vesicles, release mechanisms, and neurotransmitter receptors. Thus, reserpine depletes biogenic amines from the brain by impairing their storage in synaptic vesicles. Monoamine oxidase inhibitors block a key metabolic enzyme. Amphetamines enhance the release of catecholamines, and nearly all tricyclic antidepressants inhibit the reuptake inactivation of biogenic amines.

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During the past 15 years, the ability to measure drug and neurotransmitter receptors by simple binding techniques (first developed for studies of opiate receptors) has helped clarify actions of numerous drugs (1). Blockade of the D₂ subtype of dopamine receptors may account for the therapeutic action of a wide range of antipsychotic agents (2, 3). The same drugs appear to exert their sedative effects by blocking α_1 adrenoceptors (4). The tendency of drugs in this group to elicit extrapyramidal side effects varies inversely with their muscarinic cholinergic antagonist potency (5). Psychedelic drugs appear to act primarily by mimicking serotonin at one class of serotonin receptors, the 5-HT₂ subtype (6, 7, 8).

Recently, increased attention has been devoted to amino acid neurotransmitters as sites of psychotropic drug action. Benzodiazepines have long been known to act by facilitating synaptic effects of γ -aminobutyric acid (GABA) (9). This action involves subtle interactions between subunits of the GABA receptor that have been characterized in detail by molecular cloning studies (10). GABA and benzodiazepines bind to different sites and possibly different subunits of the receptor protein. The binding of benzodiazepines increases the ability of GABA to interact with its recognition site. Barbiturates, meprobamate, and alcohol all appear to act through the GABA receptor protein complex but at different sites than the benzodiazepines or GABA itself. In contrast to these sedating and anticonvulsant drugs, agents that block GABA receptors cause excitation and convulsions. This is true for bicuculline, which blocks the GABA recognition site. The convulsant picrotoxin blocks GABA transmission but not at the GABA recognition site. Instead, picrotoxin acts at a site related to the chloride ion channel portion of the GABA receptor protein. GABA exerts its inhibitory, hyperpolarizing synaptic effects by opening chloride ion channels in synaptic membranes. Interestingly, the chloride ion channel is part of the same receptor protein complex as the GABA recognition site. Molecular cloning studies have shown that the nicotinic acetylcholine receptor and its associated cation channel are also part of the same protein complex (11) as the inhibitory glycine receptor and its chloride ion channel (12).

These GABA, glycine, and acetylcholine receptors represent a group of neurotransmitter receptors that directly influence ion channels. Many other neurotransmitters, perhaps the majority of the biogenic amines and neuropeptides, act through biochemical second messenger systems that enzymatically amplify the signal generated by the neurotransmitter-receptor binding events. Following a chain of molecular events, neurotransmitters that act through these second messengers may ultimately open or close ion channels in the plasma membrane or influence neuronal excitability through other intracellular metabolic events. During the past 15 years, neurotransmitter receptors have been extensively characterized at a molecular

level and the drug actions mediated by these receptors have been thoroughly explored. Recent progress in understanding second messenger systems has clarified our understanding of neurotransmitter actions and focused attention on these systems as sites of psychotropic drug action.

This review will emphasize the phosphoinositide system, a major second messenger system in the brain, which has been extensively elucidated only in the last few years and which may be involved in the therapeutic actions of lithium. To introduce several general features of second messenger systems we will also outline briefly the organization of the cAMP system and focus on the role of GTP binding proteins that form the interface between transmitter receptors and both the phosphoinositide and cAMP systems.

cAMP and GTP Binding Proteins

Second messenger systems are triggered by neurotransmitter receptor stimulation (see Figure 16–1). The transmitter-receptor complex then interacts with a GTP binding protein, usually designated as a G-protein. Interaction of the G-protein with the receptor increases its affinity for GTP. For the cAMP system, activation of the G-protein in this manner enables it to dissociate from the receptor and stimulate the enzyme adenylate cyclase, enhancing synthesis of cAMP from ATP. In turn, cAMP activates a phosphorylating enzyme called cAMP-dependent protein kinase or simply protein kinase A. This kinase then phosphorylates a wide range of substrate proteins that regulate diverse cellular functions.

Some transmitters, such as catecholamines acting at β -adrenoceptors, stimulate adenylate cyclase, while others, such as enkephalin acting at opiate receptors, inhibit the enzyme. Distinct G-proteins mediate these two effects, G_s for stimulation and G_i for inhibition of adenylate cyclase (13). These differential effects involve the subunits of G-proteins. G-proteins consist of three subunits, designated alpha, beta, and gamma (14). Adenylate cyclase is directly activated by the alpha subunit of G_s . When alpha is bound to beta and gamma subunits, the G-protein is inactive. When the transmitter-receptor complex binds to the G-protein, GTP is enabled to interact potently with the G-protein, triggering dissociation of the beta and gamma subunits from the G_s protein. The resultant free alpha subunit then activates adenylate cyclase. At this point the alpha subunit, which also possesses intrinsic GTPase activity, cleaves GTP to GDP, whereupon the beta and gamma subunits reassociate with the alpha subunit, terminating activation of adenylate cyclase.

The way in which G_i inhibits adenylate cyclase has not been established definitively. However, one prominent model suggests that the initial events parallel activation of G_s and that the beta and gamma subunits dissociate from the alpha subunit when neurotransmitter receptor activation takes

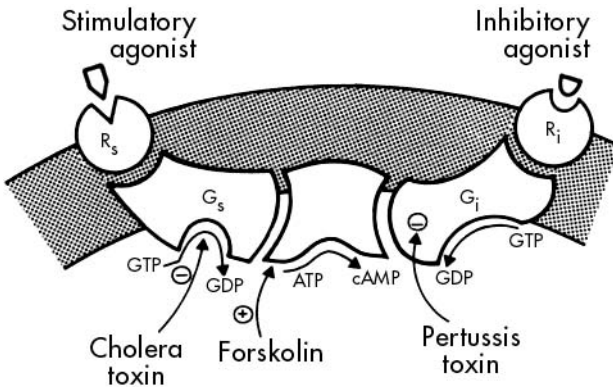


FIGURE 16-1. Bidirectional regulation of adenylate cyclase.

A stimulatory agonist is recognized by a receptor (R_s), and together they activate the stimulatory GTP-binding regulatory unit for adenylate cyclase (G_s). Activation of G_s is associated with its binding of GTP, which displaces GDP. A parallel system is shown as inhibiting adenylate cyclase by activating the inhibitory regulatory unit for adenylate cyclase (G_i). Cholera toxin blocks the GTPase activity of G_s , causing its persistent activation. Pertussis toxin blocks the inhibitory action of G_i . At low concentrations, forskolin enhances the stimulatory interaction of G_s and adenylate cyclase; at higher concentrations, forskolin directly activates adenylate cyclase.

place. However, since the concentration of G_i is substantially greater than G_s , the beta and gamma subunits released from G_i are free to complex with the alpha subunit of G_s , preventing it from stimulating adenylate cyclase. Thus, transmitters that inhibit adenylate cyclase may do so indirectly, by preventing the activation of the enzyme by G_s . Another model suggests that the alpha subunit of G_i directly inhibits adenylate cyclase (14).

Besides regulating adenylate cyclase activity, G-proteins can directly influence ion channels. In the heart, acetylcholine through muscarinic receptors slows pacemaker cell firing by opening potassium channels. Patch-clamp recordings of individual potassium channels in the heart indicate that a unique G-protein, designated G_k , opens potassium channels without any other intervening metabolic steps (15, 16). Similarly, G-proteins may directly affect calcium channels in certain cell types in the dorsal root ganglion (17, 18). The alpha subunit of the G-protein is thought to interact with the ion channels and the beta and gamma subunits are thought to serve only to inactivate alpha subunits. However, some recent studies suggested that beta subunits can themselves have direct messenger functions at potassium channels (19) or at the prostaglandin precursor, forming enzyme phospholipase A_2 (20).

Besides the three G-proteins already mentioned (G_s , G_i , and G_k), molecular cloning techniques have revealed at least four others and many more may exist

(21). One reason researchers formerly doubted the feasibility of drugs targeted at second messenger systems is that only a few principal second messenger systems have been identified, in contrast to the hundred or more distinct neurotransmitters and receptors. If only two or three second messenger systems mediate the bulk of neurotransmitter actions, drugs acting through second messengers might exert diffuse, global effects that would not be therapeutically useful. However, the considerable heterogeneity of the G-proteins and other aspects of second messengers suggest that selective drugs can be developed.

Improved understanding of the different G-proteins may indicate how they affect different brain functions discretely. The three best studied G-proteins in the brain are G_s , G_i , and G_o , a protein whose function is not yet known. Although G_s has been best characterized, it is least abundant, present at only half the levels of G_i and one-tenth the levels of G_o (22). Discrete localizations throughout the brain of G-proteins and other components of second messenger systems, to be discussed below, have considerably clarified these functions.

Although no therapeutic drugs have yet emerged in the cAMP system, some agents provide useful research probes. Forskolin is an alkaloid extracted from an Indian herb, *Coleus forskohlii*, which has been used for centuries to treat heart disease, insomnia, and convulsions. Forskolin potently activates adenylate cyclase (23), so that biological functions influenced by forskolin are candidates for being mediated by cAMP. Cholera toxin irreversibly activates adenylate cyclase by selectively modifying G_s enzymatically. By contrast, pertussis toxin selectively modifies G_i and G_o but not G_s (14).

The Phosphoinositide System

In contrast to the more than three decades of research on the cAMP system, the phosphoinositide cycle has been widely appreciated as a major second messenger system only in the past 5 years (24, 25, 26). The two systems display several similarities. They both work through G-proteins as well as phosphorylating enzymes. Both display considerable heterogeneity in their individual molecular components, so that subtle regulation in discrete parts of the brain is achievable.

A variety of receptors can trigger the phosphoinositide cycle. It is difficult to tabulate those neurotransmitters which act through adenylate cyclase versus those which act through phosphoinositides because most neurotransmitters act through both. The discrimination comes in the receptor subtypes involved. For instance, norepinephrine acting at β -adrenoceptors stimulates adenylate cyclase, but norepinephrine inhibits the enzyme through α_2 adrenoceptors and stimulates the phosphoinositide cycle through α_1 receptors (see Figure 16–2).

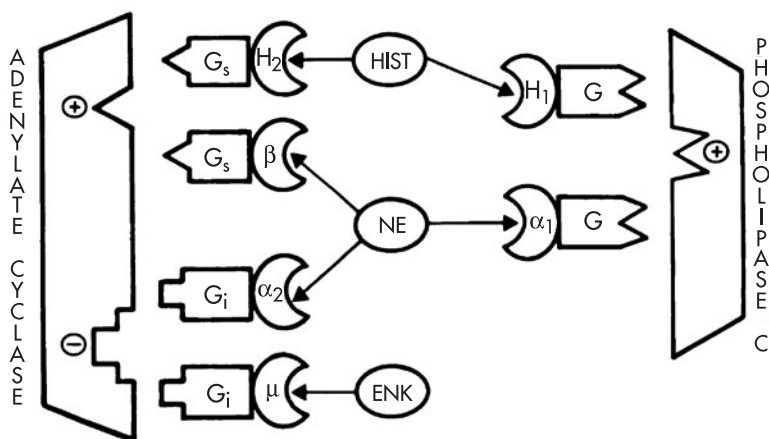


FIGURE 16-2. The multiple actions of individual neurotransmitters on second messenger systems.

These actions are carried out by their receptor subtypes, which are coupled with specific G-proteins. Histamine (HIST) stimulates adenylate cyclase by acting at H₂ receptors coupled to its stimulatory G-protein (G_s). Histamine also stimulates phospholipase C of the phosphoinositide second messenger system by acting at H₁ receptors linked to a distinct G-protein. Similarly, norepinephrine (NE) can stimulate (β receptor) or inhibit (α₂ receptor) adenylate cyclase or stimulate (α₁ receptor) phospholipase C. Conversely, each G-protein pool is coupled to several receptor subtypes: G_s is coupled to H₂ and β receptors, and the inhibitory G-protein for adenylate cyclase (G_i) is coupled to α₂ receptors and μ-opiate receptors (one of the enkephalin [ENK] receptor subtypes). An as yet unidentified G-protein (G) couples H₁ receptors and α₁ receptors to phospholipase C.

Receptor stimulation activates a phospholipase C enzyme selective for phosphatidylinositol and phosphorylated derivatives of this lipid. Phosphatidylinositol, which contains one phosphate group, is a major component of cell membranes. The phospholipid that appears to play a more prominent role as a second messenger contains an additional two phosphate groups and is termed phosphatidylinositol-bis-phosphate (PIP₂) (see Figures 16-3 and 16-4). Phospholipase C triggers the breakdown of PIP₂ to the sugar phosphate inositol trisphosphate (IP₃), which contains the three phosphate groups of PIP₂. The other product of phospholipase activity is diacylglycerol (DAG). This action of phospholipase C requires a G-protein whose identity has not yet been established definitively.

Both IP₃ and DAG influence cellular function in major ways. IP₃ binds to a unique receptor on the endoplasmic reticulum within the cell to trigger the release of calcium, which in turn can regulate cell function in many dif-

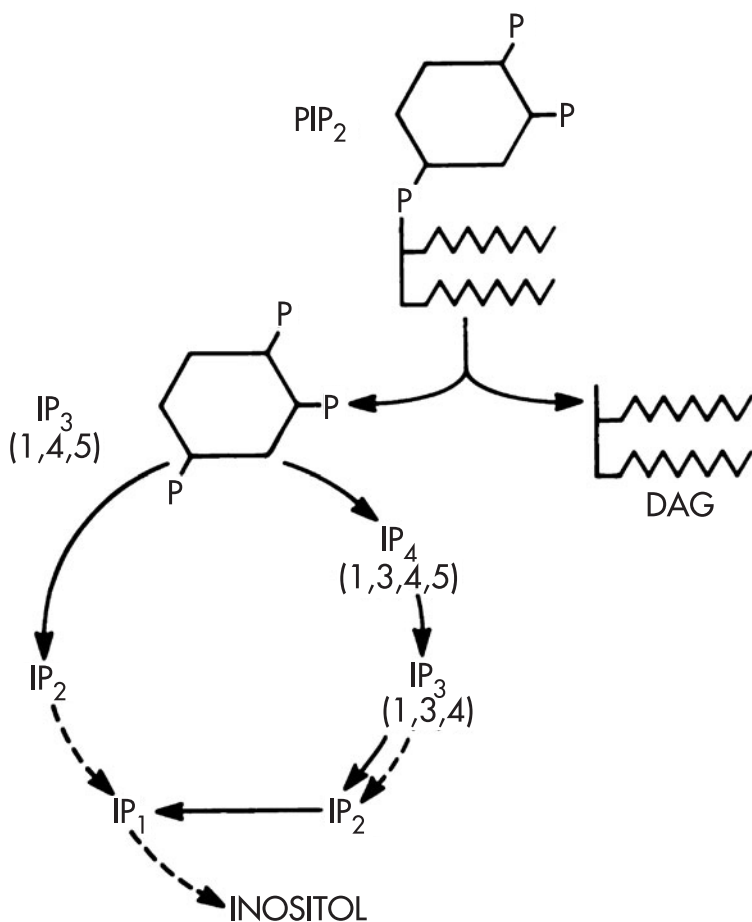


FIGURE 16-3. Inositol phosphate metabolism.

Phosphatidylinositol-bis-phosphate (PIP_2) is hydrolyzed by phospholipase C, generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3[1,4,5]$). $IP_3(1,4,5)$ is acted on by either a kinase or a phosphatase, generating 1,3,4,5-tetrakisphosphate ($IP_4[1,3,4,5]$) or inositol 1,4-bis-phosphate ($IP_2[1,4]$), respectively. $IP_4(1,3,4,5)$ is hydrolyzed to $IP_3(1,3,4)$ and then to IP_2 . Specific phosphatases hydrolyze the various IP_2 isomers to inositol. Several inositol phosphatases are inhibited by lithium. These are shown as dotted arrows. In some steps both lithium-sensitive and lithium-insensitive enzymes are involved (27) so both solid and dotted arrows are shown.

ferent ways. Thus, the IP_3 receptor is thought to be a crucial final common pathway for the phosphoinositide cycle. The IP_3 receptor represents a novel extension of the “receptor” concept because it is an intracellular recognition site in a second messenger system rather than a binding site for a neurotrans-

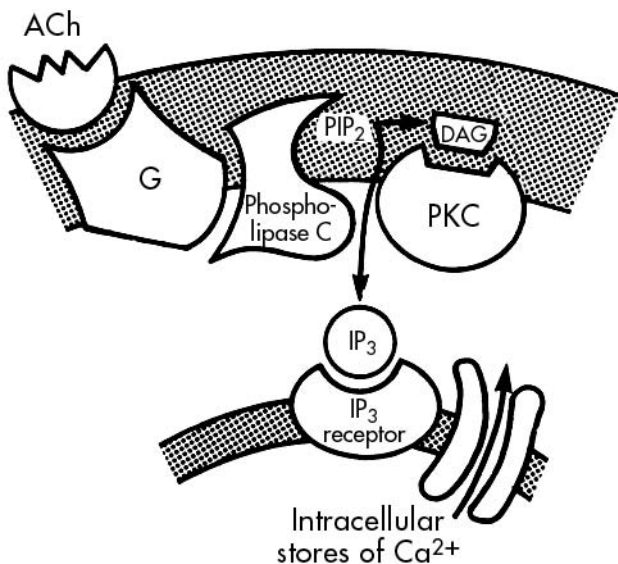


FIGURE 16-4. Receptor-mediated phosphoinositide turnover.

A transmitter such as acetylcholine (ACh) binds to its receptor, which in turn activates a G-protein (G). Activated G-protein stimulates phospholipase C, which hydrolyzes phosphatidylinositol-bis-phosphate (PIP₂), generating inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ interacts with a specific receptor and causes the release of nonmitochondrial intracellular stores of calcium. DAG activates protein kinase C (PKC), which phosphorylates a broad range of substrates. Like DAG, phorbol esters activate PKC.

mitter localized to the external surface of the plasma membrane. We (28, 29) and others (30) have been able to label IP₃ receptors through the binding of radiolabeled IP₃. We have purified the IP₃ receptor protein to homogeneity (31). The IP₃ receptor is a large protein comprising four identical subunits each of a molecular weight of about 260,000 daltons so that the full protein has a molecular weight greater than 1,000,000 daltons. When it occurs in intact membranes, binding of IP₃ to its receptors is potently inhibited by calcium in the submicromolar concentrations that normally occur within cells (see Figure 16-5). This suggests a feedback mechanism whereby calcium released by IP₃ acts back on the IP₃ receptor to block further calcium release. The ability of calcium to inhibit IP₃ binding is mediated by a protein that is distinct from the IP₃ receptor, designated "calmedin" (32). We suspect that calmedin may be an important intracellular protein regulating numerous actions of calcium, perhaps in ways similar to the well-characterized calcium binding protein calmodulin.

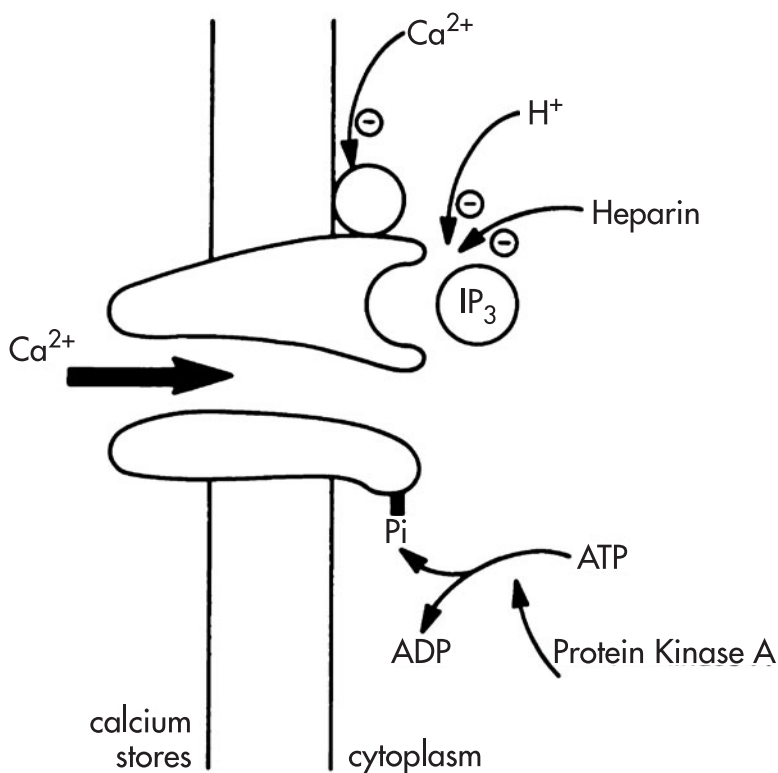


FIGURE 16-5. Schematic model of IP₃ receptor regulation.

Inositol trisphosphate (IP₃) binds to a high-affinity binding site on the cytoplasmic face of the IP₃ receptor, which is shown as a membrane-spanning protein containing a channel for passage of calcium out of intracellular stores. Binding of IP₃ to the receptor is inhibited by heparin in a competitive fashion and enhanced at slightly alkaline pH. Inhibition of IP₃ binding by calcium appears to be mediated by a distinct membrane-associated protein. Activation of the cAMP-dependent protein kinase leads to phosphorylation of the receptor protein.

The IP₃ receptor is selectively phosphorylated by cAMP-dependent protein kinase decreasing the potency of IP₃ in releasing calcium (33). This suggests a specific mechanism for “cross talk” between the cAMP and the phosphoinositide second messenger systems. In other words, cAMP formed in response to neurotransmitter action would modulate the actions of transmitters that act through the phosphoinositide system by phosphorylating the IP₃ receptor and altering its function.

IP₃ formed by hydrolysis of PIP₂ can be phosphorylated further to inositol 1,3,4,5-tetrakisphosphate (IP₄) (34, 35). The exact function of IP₄ is

not as well characterized as that of IP_3 . IP_4 may facilitate the entry of calcium into the cell through the plasma membrane possibly directly into a pool of calcium that is susceptible to release by IP_3 . Alternatively, IP_4 may trigger a movement of calcium within the cell into IP_3 -releasable pools (36). Recently, inositol 1,3,4,5,6-pentakisphosphate (IP_5) has been demonstrated in rat brain and shown to have pharmacological actions suggesting a neurotransmitter role (37).

DAG represents the second "limb" of the phosphoinositide system. Elegant studies by Nishizuka (38) have clarified the biological role of DAG, which involves the activation of protein kinase C. Protein kinase C is a phosphorylating enzyme that is dependent on calcium and phospholipids for its activity and is distinct from protein kinase A, the cAMP-dependent protein kinase. Protein kinase C within the cytoplasm moves to the cell membrane when calcium is elevated within the cell, in part by IP_3 -induced release of calcium, or with a rise in DAG, a lipophilic chemical that remains in the cell membrane after its formation from membrane phosphoinositides. DAG activates protein kinase C by enhancing its sensitivity to activation by calcium ions.

Among the numerous proteins that can be phosphorylated by protein kinase C are proteins involved in signal transduction. These substrates include receptors for a wide variety of signaling molecules such as those for acetylcholine, insulin, and epidermal growth factor, transmitter-synthesizing enzymes such as tyrosine hydroxylase, and certain G-proteins such as G_i (38, 39, 40).

Protein kinase C exerts prominent feedback effects on several aspects of the phosphoinositide system. It inhibits the ability of transmitters to produce IP_3 (41, 42). In addition, protein kinase C phosphorylates the IP_3 phosphatase enzyme, which degrades IP_3 and thus increases its enzyme activity (43, 44). Protein kinase C even phosphorylates itself, enhancing its sensitivity to calcium, thus producing a feed-forward regulation of its own activity (45).

A direct role for protein kinase C in neurotransmitter actions has been established in various excitable tissues. In our own laboratory, we first showed protein kinase C to regulate cholinergic mechanisms in smooth muscle (46). In the hippocampus, protein kinase C activation blocks a calcium-activated potassium channel, which leads to increased neuronal excitability (47). Protein kinase C also blocks the prominent inhibitory actions of several neurotransmitters, such as serotonin and adenosine (48). The effects of protein kinase C on calcium-activated potassium channels (49) and on adenosine inhibition are also produced by acetylcholine acting through muscarinic receptors. Thus, these effects of acetylcholine presumably involve the phosphoinositide system and protein kinase C.

Heterogeneous Localization of Second Messenger Systems in the Brain

Since the major second messenger systems interact with many of the same neurotransmitters, one would tend to assume that the two systems are ubiquitously distributed throughout the brain. However, although a given second messenger might be present in virtually every cell in the brain, its levels may be orders of magnitude greater in some than in others. Moreover, the existence of multiple subtypes for the various proteins involved in each second messenger system provides other opportunities for regional variations. Molecular cloning techniques have revealed marked biochemical heterogeneity of key components of the phosphoinositide system: seven distinct protein kinase C enzyme subtypes and five of phospholipase C have been identified (50, 51). Differences in their localization, and potential differences in their regulation, may provide yet another means for selective drug action on the phosphoinositide system. Direct investigations have supported the existence of regional specificity and have identified substantial differences in the localizations of components in both the cAMP and phosphoinositide systems.

We localized adenylate cyclase throughout the brain by using [^3H]forskolin, which binds to the enzyme selectively (52). Forskolin binding was localized by autoradiography, a technique in which the radioactive agent bound to thin frozen sections of the brain is visualized. In this technique sections are exposed to photographic emulsion so that development of silver grains occurs wherever the radioactive probe is bound to receptors. We observed striking differences among brain regions. Densities of adenylate cyclase in the caudate and olfactory tubercle were much greater than they were in most other brain areas. In these areas, the adenylate cyclase was contained in the cell bodies of neurons that descend to terminate in the substantia nigra, since lesions of the caudate abolished forskolin binding in the substantia nigra. These cells are recipients of the largest dopamine neuronal input in the brain. Since D_1 dopamine receptors, which are linked to adenylate cyclase activity, are also concentrated in these areas, the high levels of adenylate cyclase in the caudate olfactory tubercle are probably linked to dopamine receptors.

Several different elements of the phosphoinositide system have been localized in the brain. Our first studies visualized protein kinase C throughout the brain by autoradiography using a tritiated phorbol ester. Phorbol esters are chemicals extracted from croton oil that trigger inflammation and promote tumor development in extremely low concentrations (53). Phorbol esters bind to and activate protein kinase C in similarly low concentrations (54, 55, 56). We found striking differences in the densities of protein

kinase C throughout the brain (52, 57). In addition, the anatomical pattern observed for protein kinase C differs greatly from the localization of adenylate cyclase.

Recent studies revealed differences in the localizations of subtypes of protein kinase C (50). Four of the seven subtypes of protein kinase C have been physically separated and measured in different areas of the brain. mRNAs for three types of protein kinase C have also been visualized by *in situ* hybridization showing differential distribution of the different subtypes among neuronal populations.

At least four distinct forms of phospholipase C have been separated and molecularly cloned (51, 58, 59). We have visualized and localized mRNA for these four enzyme subtypes by *in situ* hybridization, which has revealed strikingly distinct localizations that correlate with specific neurotransmitter receptors (60). For instance, mRNA for the enzyme form designated type A (60) or form I (51) is selectively concentrated in the choroid plexus and the serotonin-containing raphe nuclei, implying a link to serotonin 5-HT-1c receptors (60). This same enzyme subtype is localized to Purkinje cells of the cerebellum, where it presumably mediates the major glutaminergic synaptic input to Purkinje cells from the granule cells (61).

We do not fully understand the functional implications of high levels of adenylate cyclase and phosphoinositide system markers in particular neuronal populations. Nevertheless, by reflecting on the maps of localizations of the two second messenger systems, it might be possible to conceptualize characteristics of brain function that are attributable to one or the other system. For example, since protein kinase C has been implicated in certain forms of synaptic plasticity (i.e., long-term changes in synaptic responses), neurons containing high levels of this pathway may be more versatile in regulating their excitability. Conceivably, insights into the response characteristics of brain systems will be possible by focusing on their repertoire of second messengers.

Within the phosphoinositide system, protein kinase C represents one limb that may be quite distinct from the inositol phosphate component. Therefore, although the activity of phospholipase C on PIP_2 generates both DAG and IP_3 , DAG may be formed to a much greater extent from phosphatidylinositol or other phospholipids such as phosphatidylcholine, which occur in very high levels in the brain, than from PIP_2 , whose levels are much lower. IP_3 , on the other hand, can be derived only from PIP_2 . To obtain insight into possible differences in the disposition of the IP_3 and DAG limbs of the phosphoinositide cycle, we localized IP_3 receptors by autoradiography (29, 62).

In general, IP_3 receptors show a similar distribution to that of protein kinase C. For instance, both protein kinase C and IP_3 receptors occur in neurons projecting from the caudate to the substantia nigra and in Purkinje

cells of the cerebellum. However, there are some marked regional variations in relative abundance. In the cerebellum, concentrations of protein kinase C and IP_3 receptors are similar. However, in the cerebral cortex and corpus striatum, protein kinase C densities are about 10 times those of IP_3 receptors. Even more dramatic, the substantia gelatinosa of the spinal cord, where sensory afferent fibers terminate, displays an extremely dense band of protein kinase C but is devoid of IP_3 receptors. Similarly, the external plexiform layer of the olfactory bulb, which contains synaptic interactions of incoming olfactory neurons, possesses very high densities of protein kinase C without detectable IP_3 receptors. Accordingly, in these latter two regions it is likely that DAG is formed from phosphatidylinositol with no substantial formation of IP_3 and few IP_3 receptors.

Using antibodies raised against the purified IP_3 receptor protein, we have successfully visualized IP_3 receptors by immunohistochemistry (C. Ross, personal communication). Localization at the light microscopic level reveals essentially the same distributions of IP_3 receptors as were observed by autoradiography with $[^3H]IP_3$. At the electron microscopic level, IP_3 receptors can be localized intracellularly to a discrete part of the endoplasmic reticulum, confirming earlier studies that this intracellular compartment contains IP_3 -sensitive stores of calcium.

Lithium: Potential Actions Through Inositol Phosphatases and G-proteins

Clarifying the therapeutic actions of lithium in psychiatry has proved far more difficult than for many other psychotropic drugs. There are several reasons. Most drugs occur in homologous series of chemical structures that vary in their therapeutic potencies. If a molecular action shows a similar variation, one can feel more confident with a relationship between the biochemical and therapeutic effects. For example, the close correlation between blockade of dopamine D_2 receptors and therapeutic potency in a large series of neuroleptics established a convincing link between dopamine receptor blockade and antipsychotic activity (2, 3). In the case of lithium no such series exists.

Lithium is also intrinsically difficult to understand, since it often relieves both manic and depressive symptoms as well as prevents recurrence of both poles of manic-depressive illness. One can readily conceptualize how a drug such as a neuroleptic can influence receptors for a given neurotransmitter to diminish manic symptoms. Similarly, facilitating the actions of certain biogenic amines may explain antidepressant drug effects. In theory, one might postulate that lithium exerts separate and distinct actions on different neurotransmitter systems to relieve mania or depression. However, abundant

clinical evidence suggests that the effects of lithium on the two poles of the illness involve similar mechanisms, since they seem to go hand in hand in individual patients and across patient groups. Indeed, the action of lithium is usually regarded as a "normalizing" effect, preventing excessive excursion away from a euthymic mood level (63).

Actions on a second messenger system might well explain the normalizing effects of lithium on mood. One neurotransmitter system might be hyperactive in mania and another one hyperactive in depression. Through a second messenger system, lithium could block movements of either neurotransmitter system into a hyperactive state. Since second messenger systems are intimately involved in mediating neuronal responsiveness to monoamine and cholinergic systems hypothesized to be involved in affective disorders (64), actions of lithium on the phosphoinositide system and on G-proteins discussed later in this paper may be relevant.

One aspect of the phosphoinositide metabolism not reviewed until now has been the mechanism whereby the phosphate groups are sequentially removed from inositol phosphates (see Figure 16-3). $IP_3(1,4,5)$ is inactivated by a phosphatase that removes the phosphate in position 5. IP_4 is also metabolized by a phosphatase that removes the 5 phosphate. This gives rise to an isomer of IP_3 , $IP_3(1,3,4)$, which is different from the active $IP_3(1,4,5)$. The $IP_3(1,3,4)$ has no known biological activity and is thought merely to be a breakdown product. It is metabolized further to isomers of IP_2 that are distinct from that formed from the breakdown of $IP_3(1,4,5)$. All of the various IP_2 isomers are metabolized to corresponding isomers of IP_1 , and these in turn are degraded to free inositol. Inositol is then attached to phosphatidic acid to form phosphatidylinositol. Phosphatidylinositol is then sequentially phosphorylated to PIP_2 .

A team of investigators at Washington University beginning in 1971 showed that lithium administration to rats depletes brain levels of inositol due to an increase in IP_1 levels, which in turn derives from a selective inhibition of the IP_1 phosphatase (65, 66). With the emergence of the phosphoinositide cycle as a second messenger system, Berridge et al. (67) suggested that lithium's psychoactive effects involve depletion of inositol to slow down the phosphoinositide cycle by preventing the formation of PIP_2 and thereafter IP_3 . The extent of "shortfall" of inositol mediators would be proportional to the overall activity of the cycle. One would predict a dampening of synaptic activity associated with the phosphoinositide cycle, particularly for those synaptic systems which are overactive. This formulation would fit nicely with the types of effects needed to explain the normalizing action of lithium.

Studies measuring the biochemical turnover of phosphoinositides and levels of inositol phosphates as a function of lithium treatment have provided evidence that lithium clearly affects inositol phosphate metabolism

(68, 69, 70, 71, 72, 73, 74, 75). To assess the physiological significance of these observations, we felt it would be important to investigate a synaptic system in the brain directly to determine whether therapeutic concentrations of lithium would influence synaptic activity in the predicted direction.

We took advantage of the system whereby acetylcholine, through muscarinic cholinergic receptors, activates phosphoinositide turnover in slices of the hippocampus (48). In this system, cholinergic agonists that act through the phosphoinositide cycle block synaptic inhibition caused by adenosine. This effect involves protein kinase C because it is mimicked faithfully by phorbol esters.

In this model system, we elicited cholinergic stimulation by applying the cholinergic agonist carbachol and demonstrated that therapeutic concentrations of lithium impair the ability of carbachol to block adenosine (76). The effect of lithium was apparent at 0.5 mM and near maximal at 1 mM of lithium, corresponding to therapeutic concentrations. We observed ionic selectivity, since rubidium had no effect. If lithium were to act by affecting phosphoinositide turnover, this effect should occur before protein kinase C activation. We would predict that lithium should not block the effects of phorbol esters. Indeed, at a time when carbachol effects were abolished by lithium, phorbol esters still reversed the effect of adenosine.

In an earlier study we showed that therapeutic concentrations of lithium influenced neurotransmitter-induced smooth muscle contraction elicited by histamine or cholinergic receptors by acting through the phosphoinositide cycle in smooth muscle (77). This result may also reflect a diminished activation of protein kinase C.

The changes in synaptic transmission we observed with lithium certainly take place through the phosphoinositide system. However, we do not have any direct evidence that inhibition of inositol phosphatases is the mechanism involved. An alternative way in which one could influence the phosphoinositide or other second messenger systems would be through G-proteins. If lithium were to diminish the coupling between G-proteins and receptors, it would also dampen excessive excursion of synaptic transmission involving any transmitter that acts through G-proteins. Avissar et al. (78) have obtained evidence for this type of action. They measured the interactions between receptors and G-proteins in two ways. In one system they measured the ability of carbachol or the β -adrenergic agonist isoproterenol to influence [^3H]GTP binding to brain membranes. In another, they monitored the influence of GTP on the competition by muscarinic agonists for radioligand binding to muscarinic cholinergic receptors. In this system, GTP acting through a G-protein decreased the potency of agonists in competing for receptor binding. Using both systems, lithium decreased apparent interactions of neurotransmitter receptors with G-proteins. Similar results were evident in intact rats

administered lithium for 12–21 days to produce therapeutic blood levels of 0.6–1.0 mM. These results suggest that lithium interferes with the activity of G-proteins, perhaps by competing with magnesium ions, which are essential for the binding of GTP to G-proteins. Recent studies indicate that lithium's effect on G-proteins may influence phospholipase C activity (79, 80).

These animal studies raise the possibility that effects of lithium on second messenger systems, such as the phosphoinositide system, may contribute to the therapeutic effects of lithium. This suggestion can be tested directly. One could design a series of organic chemical drug-like structures that potentially inhibit IP_1 phosphatase or phospholipase C. Such agents may have the therapeutic benefits of lithium but without the substantial toxicity that comes from lithium's ionic competition with sodium.

In summary, molecular characterization of second messenger systems has advanced greatly in the last few years. Like the great multiplicity of neurotransmitters and their receptors, second messenger systems, especially the phosphoinositide system, display marked heterogeneity with multiple subtypes of regulatory proteins. It is likely that new psychoactive agents will emerge from the tailoring of molecules for proteins that are key components of these second messenger systems. Studies of the actions of lithium already suggest promising directions.

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CHAPTER 17

Purified Inositol 1,4,5-Trisphosphate Receptor Mediates Calcium Flux in Reconstituted Lipid Vesicles

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Inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], a second messenger molecule involved in actions of neurotransmitters, hormones, and growth factors, releases calcium from vesicular nonmitochondrial intracellular stores (1). An $\text{Ins}(1,4,5)\text{P}_3$ binding protein, purified from brain membranes (2), has been shown to be phosphorylated by cyclic-AMP-dependent protein kinase (3) and localized by immunohistochemical techniques to intracellular particles associated with the endoplasmic reticulum (4). Although the specificity of the $\text{Ins}(1,4,5)\text{P}_3$ binding protein for inositol phosphates and the high affinity of the protein for $\text{Ins}(1,4,5)\text{P}_3$ indicate that it is a physiological $\text{Ins}(1,4,5)\text{P}_3$ receptor mediating calcium release, direct evidence for this has been difficult to obtain. Also, it is unclear whether a single protein mediates both the recognition of $\text{Ins}(1,4,5)\text{P}_3$ and calcium transport or whether these two functions involve two or more distinct proteins. In the present study we report reconstitution of the purified $\text{Ins}(1,4,5)\text{P}_3$ binding protein into lipid vesi-

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cles. We show that $\text{Ins}(1,4,5)\text{P}_3$ and other inositol phosphates stimulate calcium flux in the reconstituted vesicles with potencies and specificities that match the calcium releasing actions of $\text{Ins}(1,4,5)\text{P}_3$. These results indicate that the purified $\text{Ins}(1,4,5)\text{P}_3$ binding protein is a physiological receptor responsible for calcium release.

The $\text{Ins}(1,4,5)\text{P}_3$ receptor protein was solubilized using CHAPS as a detergent, rather than Triton X-100 which has been used previously (2), because Triton detergent is not compatible with the dialysis technique of vesicle reconstitution (5, 6). The $\text{Ins}(1,4,5)\text{P}_3$ receptor protein was purified as previously described (2) with minor modifications (see Figure 17-1). In the reconstitution experiments purified $\text{Ins}(1,4,5)\text{P}_3$ receptor protein was added to a mixture of CHAPS, solubilized phosphatidylcholine, and phosphatidylserine, and the mixed micelles of protein and lipid were dialyzed for 48 hr against a 1,000-fold excess of buffer. To determine whether the $\text{Ins}(1,4,5)\text{P}_3$ binding protein was incorporated into vesicles, we fractionated the vesicles on a continuous sucrose gradient and monitored $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding as well as levels of $[^{14}\text{C}]\text{phosphatidylcholine}$, which we used as a marker for the lipid vesicles (Figure 17-1B). The $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding activity and $[^{14}\text{C}]\text{phosphatidylcholine}$ co-migrated on the gradient and nearly 100% of the binding activity was recovered. Total protein concentration also co-migrated with the peak of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding activity (data not shown). Pure $\text{Ins}(1,4,5)\text{P}_3$ binding protein, dialyzed in the absence of lipid, appeared to aggregate as it migrated to the densest portion of the gradient (data not shown). Lipid vesicles without receptor protein appeared slightly less dense than the protein containing vesicles (data not shown). Analysis of protein within the vesicles by SDS-PAGE revealed a single band with a relative molecular mass of 260,000 (260K) (Figure 17-1A), the same as purified $\text{Ins}(1,4,5)\text{P}_3$ receptor protein not incorporated into vesicles (2). Thus, the reconstitution procedure results in the incorporation of $\text{Ins}(1,4,5)\text{P}_3$ binding protein into the vesicles with no change in molecular weight, and the protein retains its ability to bind $\text{Ins}(1,4,5)\text{P}_3$.

The properties of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in the reconstituted vesicles were essentially the same as those of the purified unreconstituted protein (2). Unlabeled $\text{Ins}(1,4,5)\text{P}_3$ inhibited $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding 50% at ~40 nM (Figure 17-2). Of a variety of inositol phosphates examined, $\text{Ins}(1,4,5)\text{P}_3$ was the most potent inhibitor of binding with $\text{Ins}(2,4,5)\text{P}_3$ being the next potent. Both $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were much less potent inhibitors of binding, whereas other inositol phosphates examined were essentially inactive (Table 17-1). Heparin, a known inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ binding (7) and $\text{Ins}(1,4,5)\text{P}_3$ -mediated calcium release (8, 9, 10, 11, 12, 13), completely inhibited binding at $100 \mu\text{g mL}^{-1}$ (Table 17-1). As previously reported for the purified, unreconstituted $\text{Ins}(1,4,5)\text{P}_3$ binding protein (2),

calcium (1 mM) had no effect on [³H]Ins(1,4,5)P₃ binding—which was markedly influenced by pH, more than doubling as pH was increased from 7.4 to 8.8 (data not shown).

To monitor calcium flux, reconstituted vesicles were incubated with ⁴⁵Ca²⁺ in the presence or absence of various concentrations of Ins(1,4,5)P₃ or other inositol phosphates (Figures 17–2 and 17–3; Table 17–1). At 10 μM Ins(1,4,5)P₃ the rate of ⁴⁵Ca²⁺ flux was stimulated at least fivefold, being half maximal within 4 s and complete within 10 s (Figure 17–3). The very rapid kinetics of Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ influx would be expected for ion channels reconstituted in small unilamellar liposomes (6). Half maximal and maximal Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ influx required ~40 nM and 1 μM Ins(1,4,5)P₃, respectively, very similar to corresponding potencies for inhibiting [³H]Ins(1,4,5)P₃ binding (Figure 17–2). The relative effects of other inositol phosphates on ⁴⁵Ca²⁺ flux also resembled their influences on [³H]Ins(1,4,5)P₃ binding, with Ins(1,4,5)P₃ being most potent and Ins(2,4,5)P₃ the only other inositol phosphate with activity (Table 17–1). These effects resemble the relative calcium-releasing potencies of inositol phosphates in several systems (14, 15, 16). Also, the relative potencies of the inositol phosphates in mediating ⁴⁵Ca²⁺ release from cerebellar microsomes were the same as in the reconstituted vesicles (data not shown). Interestingly, whereas Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄, at concentrations of 10 and 20 μM respectively, produced significant inhibition of [³H]Ins(1,4,5)P₃ binding, they did not elicit a calcium flux (Table 17–1). Whether these results indicate that at those concentrations these substances are Ins(1,4,5)P₃ receptor antagonists is not clear. Heparin (100 μg mL⁻¹) completely blocked the ability of Ins(1,4,5)P₃ (1 μM) to stimulate a ⁴⁵Ca²⁺ flux (Table 17–1) with no effect on ⁴⁵Ca²⁺ flux in the absence of Ins(1,4,5)P₃.

Although in these experiments we examined the effects of inositol phosphates on the influx of ⁴⁵Ca²⁺ into reconstituted vesicles, in other experiments we have shown similar effects on ⁴⁵Ca²⁺ release from reconstituted vesicles preloaded with ⁴⁵Ca²⁺. Ins(1,4,5)P₃ doses of 10 μM or 1 μM released, with rapid kinetics, the ⁴⁵Ca²⁺ from the inside of vesicles preloaded by freeze thaw (see Figure 17–3 legend). When the partially purified preparations of the receptor (see Figure 17–1 legend) were reconstituted in parallel with the purified receptor, the specific activity of [³H]Ins(1,4,5)P₃ binding increased 9.3-fold after the second affinity chromatography step, whereas the specific activity of Ins(1,4,5)P₃-mediated calcium flux increased 10.3-fold. In experiments using lipid vesicles without incorporated Ins(1,4,5)P₃ receptor protein, no effects of inositol phosphates on the ⁴⁵Ca²⁺ flux were detected. Also, reconstitution of protein from column fractions containing no binding activity resulted in vesicles that had no Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ transport. To demonstrate that the intact vesicle structure

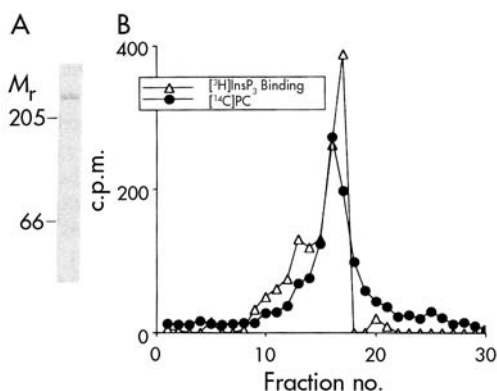


FIGURE 17-1. Reconstitution of purified Ins(1,4,5)P₃ receptor protein.

(A) SDS-PAGE analysis of protein in reconstituted lipid vesicles (7.5% gel, Coomassie Brilliant Blue stain). (B) Disposition of specific [³H]Ins(1,4,5)P₃ (open triangles) binding in continuous sucrose gradient (5–20%) fractionation of reconstituted proteoliposomes. Fraction number 1 corresponds to 20% sucrose or the bottom of the gradient, whereas fraction number 30 is 5% sucrose at the top of the gradient. The [¹⁴C]phosphatidylcholine (PC, closed circles) was used as a tracer for the lipid vesicles. Nonspecific [³H]Ins(1,4,5)P₃ binding was measured in the presence of 1 μM Ins(1,4,5)P₃. This experiment was performed three times with the same results. Binding was determined in duplicate or triplicate with replicates varying <10%.

Methods. Ins(1,4,5)P₃ receptor was purified as described (2) with the following modifications: CHAPS (1%; Sigma) was used as the detergent to allow dialysis for reconstitution, and the receptor was purified using only two affinity column chromatography steps (2). Partially purified receptor was obtained by fractionating CHAPS solubilized proteins on heparin agarose (Sigma, Type II) as described (2). The purified receptor was obtained by fractionating the proteins which elute from heparin agarose on concanavalin Sepharose (2). The reconstitution strategy was based on a previously described procedure used for the nicotinic acetylcholine receptor (6). Solubilized liposomes were prepared by sonicating (for 12–20 min in a cylindrical bath sonicator) a mixture of phosphatidylcholine (in the presence or absence of tracer amounts of [¹⁴C]phosphatidylcholine) and phosphatidylserine (Avanti Polar Lipids) at a ratio of 3:1, and a lipid concentration of 40 mg mL⁻¹ and in a buffer (buffer A) containing 50 mM NaCl, 50 mM KCl, and 20 mM Tris-HCl (pH 7.4, 25°C). Following sonication the lipid concentration was reduced to 20 mg mL⁻¹ by adding an equal volume of buffer A and CHAPS was added to bring the final detergent concentrations to 1%. This lipid mixture was then mixed with purified Ins(1,4,5)P₃ receptor (100 μg mL⁻¹ protein) or partially purified (400 μg mL⁻¹) at a ratio of 1:1. After incubation on ice for 20 min, the mixture was dialyzed (molecular mass cutoff, 10,000) against a 1,000-fold excess of buffer A supplemented with 1 mM EDTA, 1 mM EGTA, and 2 mM 2-mercaptoethanol. The buffer was changed every 8 h for 48 h to effect detergent removal and vesicle formation. These vesicles were then loaded onto a 5–20% continuous sucrose gradient (30 mL) and centrifuged overnight (SW 28, 20,000 r.p.m. [140,000g] Beckman Ultracentrifuge). Fractions (1 mL) were collected; [¹⁴C]phosphatidylcholine content and [³H]Ins(1,4,5)P₃ binding were measured. Protein concentration was determined by the method of Bradford (22), and SDS-PAGE was by the method of Laemmli (23).

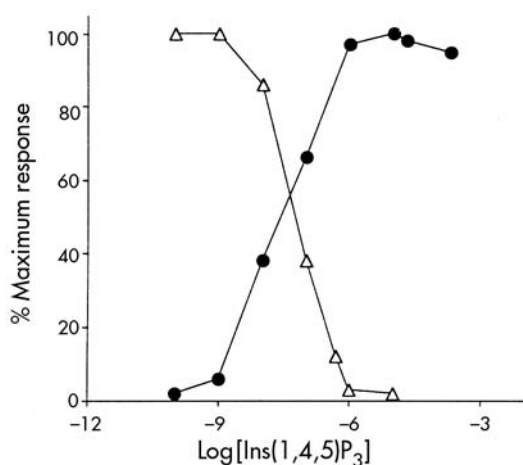


FIGURE 17-2. Concentration-response relationships for $Ins(1,4,5)P_3$ inhibition of $[^3H]Ins(1,4,5)P_3$ binding (Δ) and stimulation of $^{45}Ca^{2+}$ influx into vesicles (\bullet) reconstituted from purified $Ins(1,4,5)P_3$ receptor protein.

Calcium flux was measured as described in the legend to Figure 17-3, and the incubation time for calcium flux was 10 s. This experiment has been performed four times with essentially the same results. Measurements were made in triplicate or quadruplicate with replicates varying <10%.

is required for $Ins(1,4,5)P_3$ effects on calcium flux, we solubilized reconstituted vesicles with CHAPS (1%) and were no longer able to detect $Ins(1,4,5)P_3$ -stimulated $^{45}Ca^{2+}$ flux. In addition we examined whether the $^{45}Ca^{2+}$ accumulated in the reconstituted vesicles after $Ins(1,4,5)P_3$ stimulation could be released by ionophores. We therefore collected the vesicles after passing them over the Dowex column, incubated them in the presence and absence of the calcium ionophore A23187, and then passed them over a second Dowex column. All of the $^{45}Ca^{2+}$ accumulated after $Ins(1,4,5)P_3$ stimulation was released by the ionophore.

Our ability to reconstitute $Ins(1,4,5)P_3$ -stimulated calcium flux into lipid vesicles containing purified $Ins(1,4,5)P_3$ binding protein establishes that this protein is a physiological receptor mediating $Ins(1,4,5)P_3$ effects on calcium release. The close correlation between potency of inositol phosphates to influence ligand binding and calcium flux supports this conclusion. Our findings also establish that a single protein mediates both recognition of $Ins(1,4,5)P_3$ and the stimulation of calcium flux. In this way the $Ins(1,4,5)P_3$ receptor resembles neurotransmitter-gated ion channels, such as the nicotinic acetylcholine (6), GABA (γ -aminobutyric acid)-benzodiazepine (17), and glycine (17) receptors, in which a single protein-complex contains rec-

TABLE 17-1. [³H]Ins(1,4,5)P₃ binding and Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ flux

| Compound | Concentration (μM) | % Inhibition of [³ H]Ins(1,4,5)P ₃ binding | % Stimulation of ⁴⁵ Ca ²⁺ flux |
|----------------------------|-------------------------|---|--|
| Ins(1,4,5)P ₃ | 1 | 100 | 97 |
| Ins(1,3,4)P ₃ | 10 | 40 | 0 |
| Ins(2,4,5)P ₃ | 2 | 72 | 52 |
| Ins(2)P | 5 | 3 | 0 |
| Ins(4)P | 5 | 0 | 0 |
| Ins(1,4)P ₂ | 10 | 5 | 0 |
| Ins(1,3,4,5)P ₄ | 20 | 62 | 0 |
| InsP ₅ | 10 | 12 | 0 |
| InsP ₆ | 10 | 15 | 0 |
| Heparin | 100 μg mL ⁻¹ | 100 | 0 |
| Ins(1,4,5)P ₃ | 1 | | |
| +heparin | 100 μg mL ⁻¹ | — | 3 |

Note. Calcium flux and [³H]Ins(1,4,5)P₃ binding were determined as described in the legends to Figures 17-1 and 17-2. Flux measurements were made after a 10 s incubation as in Figure 17-3. These experiments have been performed three times in triplicate or quadruplicate with replicates varying by <15%.

ognition sites both for the neurotransmitter and associated ion channels. These neurotransmitter receptors are comprised of several distinct subunits, whereas the Ins(1,4,5)P₃ receptor seems to be a tetramer of four identical subunits (2). The neurotransmitter receptors are integral proteins of the plasma membrane with recognition sites on the extracellular surface, whereas the Ins(1,4,5)P₃ receptor is a novel intracellular receptor with the recognition site for Ins(1,4,5)P₃ on the cytoplasmic side. In this way the Ins(1,4,5)P₃ receptor resembles the recently characterized (18, 19, 20) and cloned (21) ryanodine receptor, which mediates calcium flux from the sarcoplasmic reticulum of skeletal muscle. The Ins(1,4,5)P₃ receptor also resembles the ryanodine receptor in having a high relative molecular mass, and a similar proposed subunit structure and chromatographic behavior (18, 19, 20, 21). It is likely, therefore, that these two receptors are members of a new class of intracellular ligand-gated ion channels.

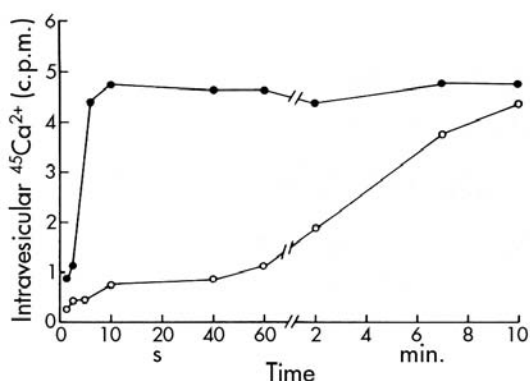


FIGURE 17-3. Time course of $^{45}\text{Ca}^{2+}$ flux in vesicles reconstituted with purified Ins(1,4,5) P_3 receptor.

Open symbols, accumulation of $^{45}\text{Ca}^{2+}$ in reconstituted vesicles in the absence of any agonist; closed symbols, $^{45}\text{Ca}^{2+}$ accumulated in the presence of 10 μM Ins(1,4,5) P_3 . Data are expressed as c.p.m. ($\times 1,000$). This experiment has been replicated three times with similar results. Measurements were made in duplicate or triplicate with replicates varying $<10\%$.

Methods. Following reconstitution of the purified Ins(1,4,5) P_3 receptor as described in the legend to Figure 17-1, vesicles were dialyzed overnight in buffer A supplemented with 2 mM 2-mercaptoethanol to remove the calcium chelators which interfere with calcium flux measurements and to bring the final calcium concentration to nominally free levels of $\sim 10 \mu\text{M}$ free calcium as measured by a calcium sensitive macroelectrode. Calcium flux was determined using tracer amounts of $^{45}\text{Ca}^{2+}$ and cation exchange chromatography. Calcium influx was measured by incubating 85 μL of reconstituted vesicles with 2 μCi $^{45}\text{Ca}^{2+}$ in the presence or absence of 10 μM Ins(1,4,5) P_3 . The final reaction volume was 100 μL . After incubation for the indicated times, the flux reaction was stopped with a threefold excess of buffer A supplemented with 0.3 mM CaCl_2 , 5 mM MgSO_4 , 1 mM CdCl_2 , and 100 $\mu\text{g mL}^{-1}$ heparin (Sigma) (stop buffer). This 400 μL mixture of vesicles and stop buffer was immediately loaded onto a 3 mL column of Tris-Dowex (Sigma, Dowex 50-W X-8, pH 8.0) which had been equilibrated with buffer containing 200 mM sucrose, 20 mM Tris (pH 7.4, 25°C), and 0.3% BSA. The column was immediately washed with 3.5 mL of the same buffer (without BSA) to wash the vesicles and the intravesicular $^{45}\text{Ca}^{2+}$ through the column. The $^{45}\text{Ca}^{2+}$ content of the vesicles was determined by liquid scintillation spectrometry. In some experiments, however, following the removal of extravesicular calcium by the Dowex column, the vesicles were immediately incubated in 3.5 mL of the sucrose containing buffer in the presence or absence of 10 μM A23187, a calcium selective ionophore. After a 10 min incubation at 22°C, the vesicles were reloaded onto (and washed through) a second Dowex column (6 mL column volume) to determine the selective releasability of the accumulated calcium. For efflux measurements vesicles were loaded with $^{45}\text{Ca}^{2+}$ by rapid freeze thaw. After freeze thaw, vesicles were diluted fivefold into buffer A in the presence or absence of 10 μM Ins(1,4,5) P_3 . After a 10 s incubation at 22°C the flux reaction was stopped and the intravesicular $^{45}\text{Ca}^{2+}$ content was determined as described above.

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Part VII

NITRIC OXIDE AS A NEUROTRANSMITTER

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COMMENTARY

Just Say "Yes": Snyder's Approach to the Difficult Problem of NO

Anne B. Young

Solomon Snyder and his colleagues were key drivers in defining the role of the gas nitric oxide as a nontraditional neurotransmitter in the nervous system. Snyder's work brought attention to the role of nitric oxide in the peripheral and central nervous systems and spurred a whole new field of neuroscience and interneuronal communication. Some of Snyder's key contributions to the field are highlighted in this commentary (1, 2, 3).

Early Observations in the Periphery

Years ago, it was found that endogenous nitrates were produced in large amounts in mammals and that their levels rose during infections (4). Nitrates and nitrites were found to arise from macrophages through oxidation of L-arginine. A reactive substance was then found that was short-lived and diffusible. It was eventually identified as nitric oxide (NO) (5, 6). Without L-arginine, the macrophage NO was not produced and the macrophages were no longer toxic to tumor cells and bacteria. Their toxicity was also blocked by L-N-methyl-arginine.

In the periphery, it was discovered that vasodilatation was due in part to a factor made by endothelial cells. This factor, endothelium-derived relaxing factor or EDRF, was absent when the endothelium was removed from vessels. Since the endothelial cells have no obvious evidence of conventional cell-to-cell signaling, other methods of communication were investigated. A

diffusible factor was identified that was made by endothelial cells and had a very short half-life. This factor was dependent on the presence of arginine and its conversion to citrulline. As with macrophages, NO was found to be a by-product of this reaction (7, 8). Inhibition of the conversion by L-N-methyl-arginine inhibited the effect of EDRE.

Subsequent studies have implicated NO action in a variety of tissues, including the pylorus and intestine, the adrenal medulla, the liver, and the bulbos cavernosus (4).

Evidence for Central Nitric Oxide Actions

Effect of Nitric Oxide on Glutamate Function

Garthwaite et al. showed in 1988 (9) that glutamate caused a large increase in cGMP in cerebellum that was dependent on a messenger similar to EDRE. Glutamate was known to be the neurotransmitter of the granule cells, the only intrinsic excitatory transmitter in the structure. Garthwaite et al. (10) and Bredt and Snyder (1; Chapter 18 in this volume) then showed in 1989 that this increase in cGMP was dependent on arginine and its conversion to citrulline. cGMP formation was inhibited by L-N-methyl-arginine and hemoglobin, known inhibitors of nitric oxide synthase (NOS). This was the first demonstration of a role for NO in mammalian brain. At the time, however, the role of cGMP in neuronal function was largely unknown. It is likely to enhance the activity of cGMP-dependent protein kinase that is selectively expressed in cerebellum. cGMP also affects various ion channels in the periphery. cGMP regulates cAMP-dependent phosphodiesterases (1, 4). NO could only function as a nontraditional neurotransmitter because it is synthesized in the cytoplasm; it is not stored in vesicles but rather diffuses rapidly to the postsynaptic neuron, where it diffuses into the postsynaptic terminal and acts on (i.e., nitrosylates) various proteins and enzymes. Unlike other neurotransmitters, it does not interact with any selective membrane receptor.

Distribution of Nitric Oxide Synthase in Brain

In 1990, Bredt and Snyder then purified NO synthase from cerebellum and developed antibodies to the enzyme (11). Purification was a feat because the enzyme had proved difficult to isolate. Bredt and Snyder figured that calcium and calmodulin might stabilize the enzyme. This proved true and allowed its subsequent purification. Localization studies showed the highest concentrations of enzyme to be in the cerebellum, olfactory bulb, dentate gyrus of hippocampus, stria terminalis, and pedunculopontine nucleus. Scattered immunoreactive medium-to-large aspiny interneurons were found in striatum and cerebral cortex. The immunoreactive cells in cerebellum were the

granule cells and basket cells that receive excitatory input from mossy fibers that end on synapses with postsynaptic NMDA receptors. Those in striatum and cortex were positive for somatostatin. All neurons were positive for NADPH-diaphorase (which has now been shown to be identical to NOS). In basket cells of the cerebellum, NOS colocalized with GABA, in granule cells with glutamate, and in pedunculopontine cells with acetylcholine. Thus NOS does not colocalize with a particular single neurotransmitter. NOS-positive cells were also found in the myenteric plexus, in nerve terminals on blood vessels, and in the adrenal medulla.

Nitric Oxide and Excitotoxicity

The question was examined of whether NO in the nervous system is toxic in the way that NO is toxic in the periphery when it is released from macrophages. A role for nitric oxide synthase in excitotoxicity came when it was found, in cultured cerebral cortex neurons, that glutamate toxicity could be modified by the addition of arginine or *N*-methyl-arginine (12). When cultures were exposed to *N*-methyl-D-aspartate, neuronal death was observed, particularly around NOS-positive neurons. NOS-positive cells themselves were resistant to the NMDA but were sensitive to quisqualate and kainate. Neurotoxicity was attenuated by *N*-methyl-arginine and by incubation in medium devoid of L-arginine. The survival of NOS-positive cells is also seen in several neurodegenerative disorders: such cells include striatal NOS interneurons in Huntington's disease and cortical NOS interneurons in Alzheimer's disease. Ischemic stroke was also found to be attenuated in mice with targeted disruption of the neuronal NOS gene (13).

Cloning and Properties of Neuronal Nitric Oxide Synthase

Protein Properties

Cloning of the gene for neuronal NOS was accomplished in 1989 by Bredt et al. The gene had an open reading frame of 4,287 bases encoding a protein of 1,429 amino acids and a molecular weight of 160K. The gene is homologous to separate genes for endothelial NOS, inducible NOS, and cytochrome P450 reductase (2; Chapter 19).

Enzymatic Modulation

NOS is involved in oxidative activity and uses several cofactors—five, in fact. There are consensus sequences for NADPH, FAD, and flavin mononucleotide (FMN) binding (2, 4). In addition, there is an iron–protoporphyrin IX binding site and a tetrahydrobiopterin site. Arginine is oxidized to *N*-hydroxy-arginine and requires NADPH and O₂. Next, *N*-hydroxy-arginine is

converted to citrulline and this step also requires NADPH and O_2 . The conversion is dependent on calmodulin and calcium in addition.

The various NOS genes encode for distinct proteins that bind cofactors and substrates differentially (4). For inducible NOS, calmodulin is so tightly bound that there is no requirement for exogenous calmodulin. It is neither stimulated by calcium nor inhibited by calmodulin antagonists. For neuronal NOS, tetrahydrobiopterin is tightly bound and thus exogenous tetrahydrobiopterin is not required for enzyme activity. In the nervous system, NMDA stimulates calcium influx into the postsynaptic nerve terminal, binding to calmodulin and activating NOS. Calmodulin binding to neuronal NOS requires calcium. Via other neurotransmitters such as acetylcholine, activation triggers the phosphatide inositol pathway with increases in intracellular calcium that in turn stimulate NOS.

Homology to Other Proteins

nNOS has considerable homology to the eNOS and iNOS proteins. In the C-terminal half of each protein, there are sites for NADPH, FMN, FAD, and calmodulin binding sites (2, 4). The proteins have different properties otherwise, as cited above.

Role of Nitric Oxide in Behavior

Nelson et al. in 1995 demonstrated behavioral abnormalities in mice with targeted disruption of the neuronal NOS gene (3; Chapter 20). Male but not female NOS knockout mice display increased social aggression and inappropriate sexual behavior. These mice have lost nNOS and NADPH-diaphorase activity in brain. The animals appear normal, and pathologically, the only obvious abnormality is excessive stomach dilatation due to pyloric hypertrophy. Otherwise, no pathology can be detected.

Male NOS knockout mice display clear-cut behavioral abnormalities, however. There is a large difference in latency to first attack in males housed together. The total number of subsequent attacks was increased in the NOS-negative mice. Submissive behaviors were much reduced as well compared to wild-type mice. Nevertheless, male NOS knockout mice have normal testosterone levels, smell and motor strength, and agility.

Sexual behavior as manifested by the number of mounts per 15 minutes was increased at each time point after introduction of male to female in the NOS-negative mice compared to wild-type. There were no other abnormalities noted, and synaptic plasticity was still intact, as evidenced by normal long-term potentiation and long-term depression in these animals. NO thus appears to play a critical role in limbic function and, in particular, sexual and aggressive behavior.

Summary

NO is a unique gaseous neurotransmitter that has widespread, important activity throughout the body. In the periphery, NO regulates arterial relaxation and plays a role in cardiovascular function and endotoxic shock. NO acts as a physiological mediator of penile erection as a transmitter in NO-positive neurons innervating the corpora cavernosa and in neuronal plexuses in the adventitial layer of the penile arteries (14). NO modulates gastric motility through NO-positive myenteric plexus neurons synapsing on intestinal smooth muscle. In the nervous system it is densely expressed in cerebellum, dentate gyrus of hippocampus, olfactory bulb, pedunculopontine neurons, and cortical and striatal aspiny interneurons. NO plays a role in excitotoxic neuronal injury in stroke and neurodegenerative disease. NO plays a key role in sexual and aggressive behavior. Overall, the work of Snyder and his students opened up this whole new important and therapeutically relevant field of NO research in the nervous system.

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CHAPTER 18

Nitric Oxide Mediates Glutamate-Linked Enhancement of cGMP Levels in the Cerebellum

David S. Bredt
Solomon H. Snyder

The striking inability of blood vessels to respond to vasodilating substances in the absence of an intact endothelium was resolved by the discovery of an “endothelium-derived relaxing factor” that subsequently was shown to be identical to nitric oxide (NO) (1, 2, 3). Besides endothelial cells, NO formation has been demonstrated in macrophages (4), and indirect evidence suggests the formation of an endothelium-derived relaxing factor in brain tissue (5). An enzymatic activity forming NO from arginine has been reported in endothelial cells (6), macrophages (4), neutrophils (7), and brain homogenates (8). Direct experimental evidence has established a function for NO in relaxing the smooth muscle of blood vessels (9) and in mediating the cytotoxic effects of macrophages and neutrophils (10). A role for NO in the brain has been elusive.

Glutamate, the major excitatory neurotransmitter in the brain, acts through several receptor subtypes, some of which directly open ion channels, whereas others stimulate the inositol phospholipid cycle (11, 12) and some stimulate the formation of cGMP (13). The enhancement of cGMP

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formation by glutamate is most prominent in the cerebellum (14) where Purkinje cells possess the highest levels of cGMP (14), guanylate cyclase (15), GMP-dependent protein kinase, and its protein substrate (16).

In the present study we show a striking enhancement by glutamate and related excitatory amino acids of the conversion of arginine to NO and the associated formation of citrulline. Moreover, we show that N^{ω} -monomethyl-L-arginine (MeArg), a potent inhibitor of the enzymatic transformation of arginine to NO, blocks glutamate-elicited cGMP formation, an effect that is specifically reversible by excess arginine. These findings establish NO as a "messenger molecule" mediating glutamate synaptic actions upon cGMP.

Materials and Methods

Materials

[^3H]Arginine (53 Ci/mmol; 1 Ci=37 GBq) was obtained from DuPont/NEN. cGMP radioimmunoassay kits were obtained from Amersham. MeArg was obtained from Calbiochem. All other chemicals were obtained from Sigma. Hemoglobin was prepared from methemoglobin as described (17).

Determination of NO

NO was measured as its breakdown product, NO_2^- . Ten cerebella were homogenized in 12 mL of 0.32 M sucrose/20 mM Hepes, pH 7.2/0.5 mM EDTA/1 mM dithiothreitol and centrifuged at 20,000g 15 min, and the supernatant was passed over a 0.75-mL column of Dowex AG50WX-8 (Na^+ form) to remove endogenous arginine. Incubations were initiated by addition of 340 μL of homogenate to buffer containing (final concentrations) 2 mM NADPH, 0.45 mM Ca^{2+} (1 μM free calcium), 200 μM arginine, [^3H]arginine (1 $\mu\text{Ci/mL}$), and various concentrations of MeArg in a total volume of 400 μL . After a 45-min incubation at 37°C, [^3H]citrulline was assayed in 150- μL aliquots of the incubation mixture after separation from [^3H]arginine by cation-exchange chromatography as described below. NO_2^- concentration was determined by adding 250 μL of the incubation mixtures to 250 μL of Greiss reagent/ 5% (vol/vol) H_3PO_4 /1% sulfanilic acid/0.1% N -(1-naphthyl)-ethylenediamine. The reaction of NO_2^- with this reagent produces a pink color, which was quantified at 554 nm against standards in the same buffer.

Preparation of Brain Slices

Cerebella from 10-day-old rats were cut at 0.4-mm intervals in both the sagittal and coronal planes using a McIlwain tissue chopper. The slices were dispersed in Krebs-Henseleit buffer containing 118 mM NaCl, 4.7 mM KCl, 2 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , and

11 mM glucose. Cerebellar slices from a single litter were pooled (≈ 10 rats) and were incubated for 60 min in 250 mL of buffer continuously gassed with 95% O₂/5% CO₂ at 37°C.

cGMP Levels and [³H]Citrulline Formation in Slices

After the 1-hr preincubation, 20- μ L aliquots of gravity-packed slices were transferred to prewarmed 5-mL minivials (Beckman) containing 250 μ L of Krebs-Henseleit buffer (equilibrated with 95% O₂/5% CO₂). Appropriate concentrations of MeArg were added and all slices were further incubated 20 min under 95% O₂/5% CO₂ at 37°C. For determinations of cGMP, after a 3-min exposure to excitatory amino acids, slices were inactivated by boiling for 5 min in 1 mL of 50 mM Tris-HCl, pH 8.4/5 mM EDTA. After sonication, cGMP levels were determined by radioimmunoassay. [³H]Citrulline accumulation after a 15-min exposure of slices to [³H]arginine (3 μ Ci/mL) and the appropriate excitatory amino acid was assessed by adding 0.75 mL of ice-cold buffer with 5 mM arginine and 4 mM EDTA, whereupon the slices were centrifuged 10,000g for 1 min at 0°C, the medium was removed, and 1 mL of 1 M trichloroacetic acid was added to precipitate protein. After sonication and centrifugation, the supernatants were removed and the trichloroacetic acid was extracted three times with 2 mL of diethyl ether. Aliquots of 0.5 mL of the extract were neutralized with 2 mL of 20 mM Hepes (pH 6.0) and applied to 2-mL columns of Dowex AG50WX-8 (Na⁺ form) and material was eluted with 2 mL of water. [³H]Citrulline was quantified by liquid scintillation spectroscopy of the 4-mL flow-through, whose sole radioactive component was verified as [³H]citrulline by thin layer chromatography (data not shown).

Results

Prior to initiating experiments with cerebellar slices, we examined the conversion of [³H]arginine to [³H]citrulline in cerebellar homogenates. As has been reported (8), we found this conversion to be completely dependent on added NADPH as well as added Ca²⁺ (data not shown). Fifty percent of maximal [³H]citrulline formation was apparent at about 150 nM calcium. We also have confirmed the kinetic constants for the arginine to citrulline conversion (8). We examined the conversion of increasing concentrations of [³H]arginine to [³H]citrulline and observed a K_m of 6 μ M and V_{max} of 150 pmol per min per mg of protein. We also have examined the effects of various concentrations of MeArg on [³H]citrulline formation in cerebellar homogenates and observed a K_i for inhibition of [³H]citrulline formation of about 2 μ M (data not shown). The conversion of [³H]arginine to [³H]citrulline appears to be stoichiometric with the formation of NO. MeArg displays purely competitive inhibition of both activities (Table 18–1).

TABLE 18-1. **Stoichiometric inhibition of [³H]citrulline and NO formation by MeArg**

| MeArg, μ M | [³ H]Citrulline formation % maximum | NO formation % maximum | Calculated activity % maximum |
|----------------|--|---------------------------|----------------------------------|
| 0 | 100 | 100 | 100 |
| 10 | 83 | 82 | 81 |
| 20 | 73 | 72 | 70 |
| 50 | 53 | 51 | 49 |
| 100 | 32 | 29 | 33 |
| 200 | 23 | 21 | 20 |
| 2,000 | 0 | 0 | 2 |

Note. [³H]Citrulline and NO, measured as its breakdown product NO₂⁻, were assayed in cerebellar homogenates with 200 μ M [³H]arginine added. Calculated activities were determined assuming competitive inhibition by MeArg and the observed kinetic parameters, K_m =6 μ M for arginine and K_i =1.5 μ M for MeArg. Maximal levels of [³H]citrulline and NO formation were 10,200 cpm and 7.5 μ M, respectively. Data represent the mean of triplicate determinations and varied <8%.

NO is extraordinarily unstable with a half-life at room temperature of about 10 sec. (3). Most studies of NO formation have measured chemiluminescence of the complex formed between NO and ozone, which is a complex laborious procedure (2). We have developed a simple and sensitive procedure for monitoring the conversion of [³H]arginine to [³H]citrulline in brain slices (Figure 18-1). This conversion is dramatically enhanced by glutamate and *N*-methyl-D-aspartate (NMDA). NMDA is the most potent agent examined, producing a 2.5-fold elevation in [³H]citrulline formation at 100 μ M concentration and a maximal elevation of almost 3-fold at 1 mM concentration, as 5 mM NMDA produces no greater effect (data not shown). Glutamate is less potent than NMDA but produces the same maximal effect, while kainate produces lower enhancement with only a 1.8-fold increase in [³H]citrulline at 1 mM kainate. Quisqualate at 0.1 and 1.0 mM has minimal effect on [³H]citrulline formation.

The relative potencies of glutamate and its derivatives in stimulating cGMP formation are the same as stimulating [³H]citrulline formation, with NMDA being most potent and glutamate somewhat less potent though producing the same maximal effect. Kainate produces less of a maximal stimulation of cGMP than NMDA or glutamate, and quisqualate is least active (Figure 18-1B).

To determine whether the NMDA stimulation of NO formation and cGMP levels are linked, we compared the concentration-response effects of NMDA on [³H]citrulline and cGMP formation (Figure 18-2A). Concentra-

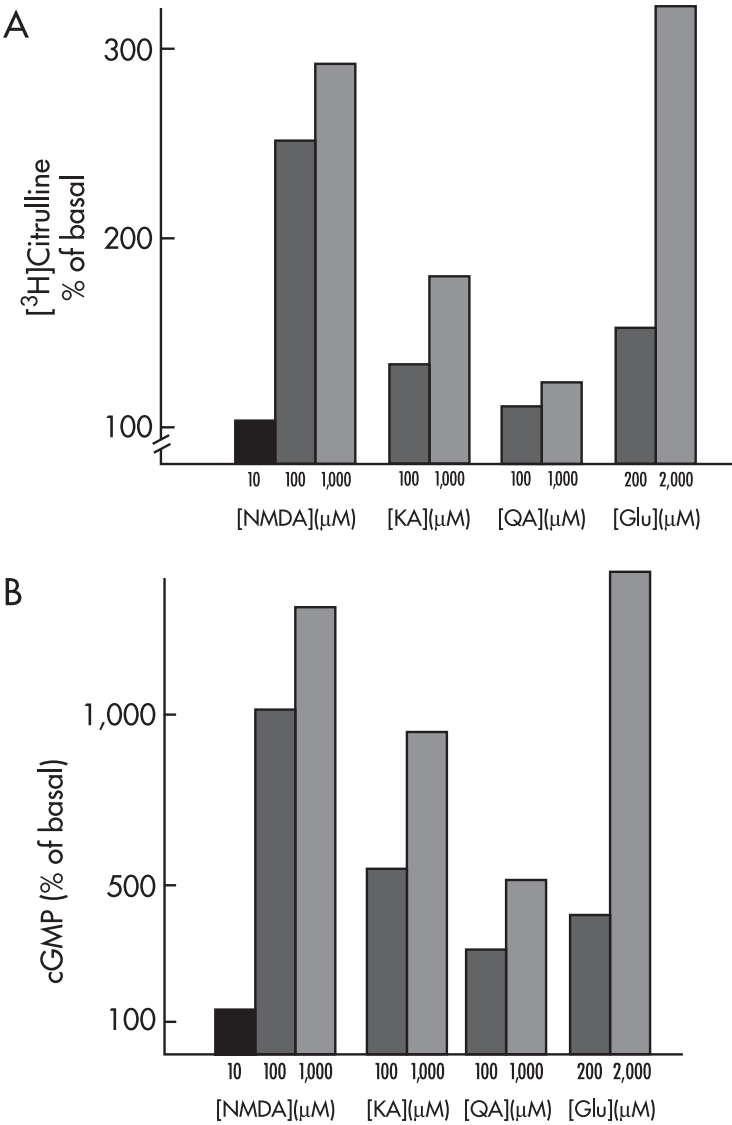


FIGURE 18-1. Pharmacology of excitatory amino acid-mediated enhancement of $[^3\text{H}]$ arginine to $[^3\text{H}]$ citrulline conversion (A) and cGMP levels in cerebellar slices (B).

Basal levels for $[^3\text{H}]$ citrulline formation were 1,640 cpm and for cGMP levels were 2.4 pmol/mg of protein. Values are the mean of triplicate determinations with a SD <15%. The experiments were repeated with similar results.

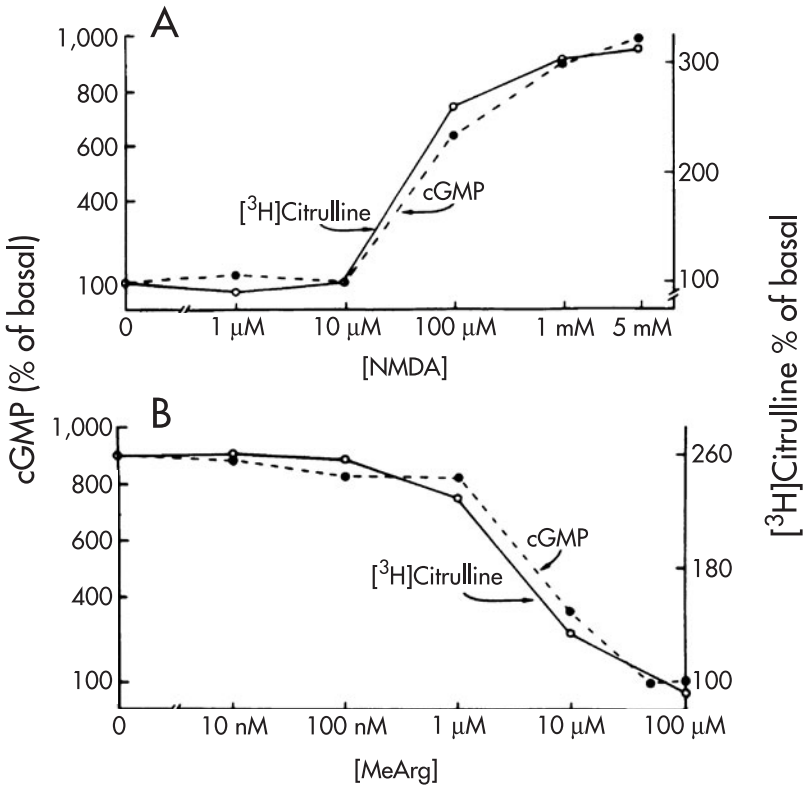


FIGURE 18-2. Concentration-response relationships for NMDA stimulation (A) and MeArg inhibition (B) of [³H]citrulline and cGMP formation in cerebellar slices.

All slices in (B) were stimulated with 500 μM NMDA. Basal levels for [³H]citrulline formation were 1,478 cpm and for cGMP levels were 2.7 pmol/mg of protein. Data are from representative experiments in triplicate with SD <15% and were replicated twice.

tion-response curves for the two are virtually superimposable with negligible effects at 10 μM NMDA, marked effects at 100 μM NMDA, and maximal effects at 1 and 5 mM NMDA.

MeArg is a potent inhibitor of the conversion of arginine to NO with an IC₅₀ in brain homogenates of about 5 μM (8). We compared the effects of MeArg on the NMDA enhancement of cGMP and [³H]citrulline formation in slices (Figure 18-2B). MeArg displays identical potencies in blocking NMDA enhancement of cGMP and [³H]citrulline formation with an IC₅₀ of about 6 μM.

If MeArg blocks NMDA stimulation of cGMP formation by competitively inhibiting the conversion of arginine to NO, its influence should be reversed

by arginine. Accordingly, we examined effects of increasing arginine concentrations on the ability of MeArg to block NMDA stimulation of cGMP formation (Figure 18–3). In the absence of MeArg no enhancement of NMDA stimulation of cGMP levels occurs with 1–1,000 μM arginine. Both 20 μM and 200 μM MeArg concentrations completely block NMDA stimulation of cGMP levels. Arginine reverses the effects of MeArg competitively. Thus 20 μM arginine provides a 50% reversal of the effects of 20 μM MeArg, and about 200 μM arginine is required to reverse by 50% the influences of 200 μM MeArg.

The competitive reversal by arginine of MeArg effects strongly suggests that MeArg actions on NMDA stimulation of cGMP levels derive from competitive inhibition of endogenous arginine. To provide further evidence for this specificity, we examined the ability of other amino acids to reverse the effects of MeArg (Figure 18–4). We evaluated leucine, lysine, ornithine, citrulline, and alanine at 1 mM concentrations. None of these amino acids are able to reverse the MeArg blockade of NMDA-stimulated cGMP formation.

To obtain more direct evidence that arginine influences on cGMP derive from the formation of NO, we examined the effects of hemoglobin and superoxide dismutase (Figure 18–5). Hemoglobin complexes with NO to block its stimulation of guanylate cyclase (18, 19), whereas superoxide dis-

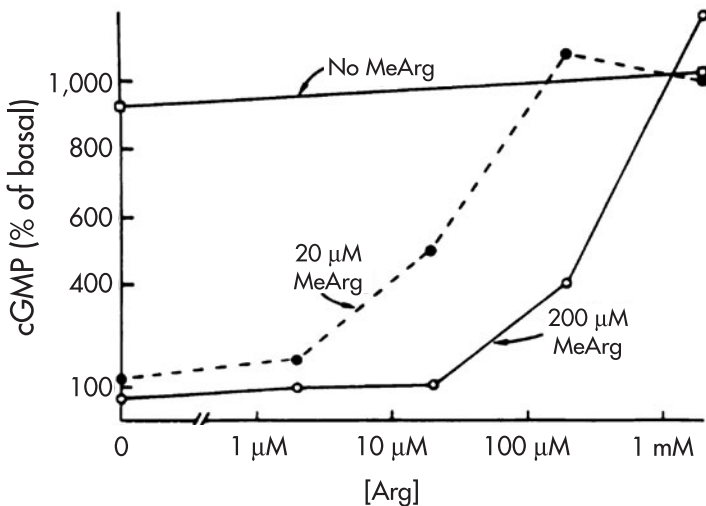


FIGURE 18–3. Reversal of MeArg inhibition of NMDA-mediated enhancement of cGMP.

All slices were stimulated with 500 μM NMDA. Basal level of cGMP was 2.5 pmol/mg of protein. Data are the mean of triplicate determinations with an SD <15%. This experiment was repeated twice.

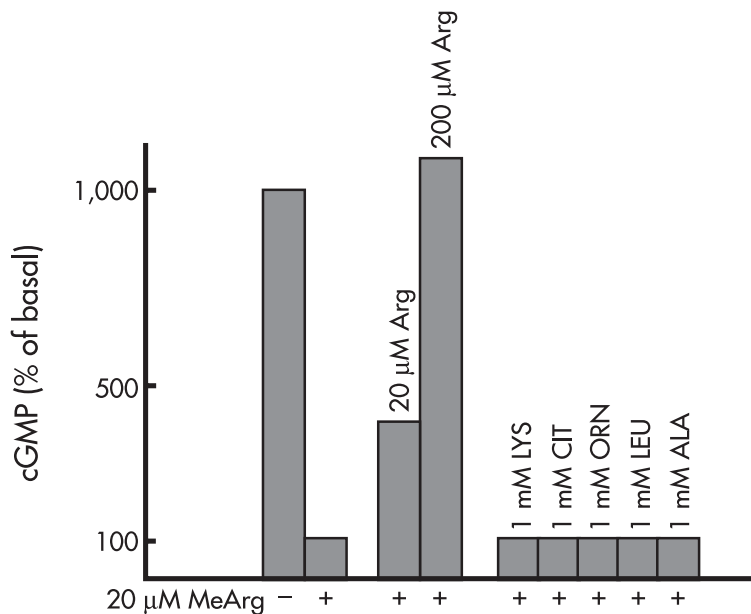


FIGURE 18-4. Specificity of arginine reversal of MeArg inhibition of cGMP accumulation.

All slices were treated with 500 μM NMDA. Basal level of cGMP was 3.1 pmol/mg of protein. All amino acids were added with MeArg 20 min prior to NMDA. Data are the mean of triplicate determinations from a representative experiment, which was repeated with similar events. +, MeArg added; –, no MeArg added; CIT, citrulline; ORN, ornithine.

mutase enhances the effects of NO on guanylate cyclase by removing superoxides, which destroy NO. Hemoglobin prevents NMDA stimulation of cGMP levels with half-maximal effects at about 6 μM, consistent with the potency of hemoglobin in binding NO (18). Superoxide dismutase enhances the ability of 50 and 500 μM NMDA to stimulate cGMP levels.

Discussion

To facilitate investigations of NO as a messenger substance, we have developed a simple and sensitive technique in which the conversion of [³H]arginine to [³H]citrulline is monitored, since the formation of citrulline is stoichiometric with the synthesis of NO. Utilizing this technique one can readily assay 100 samples in a day. The technique is highly sensitive and can easily detect as little as 1 pmol of [³H]citrulline formed in brain slices. By contrast, the formation of endogenous NO is most usually monitored by forming a complex of NO and ozone, which is then detected by chemilumi-

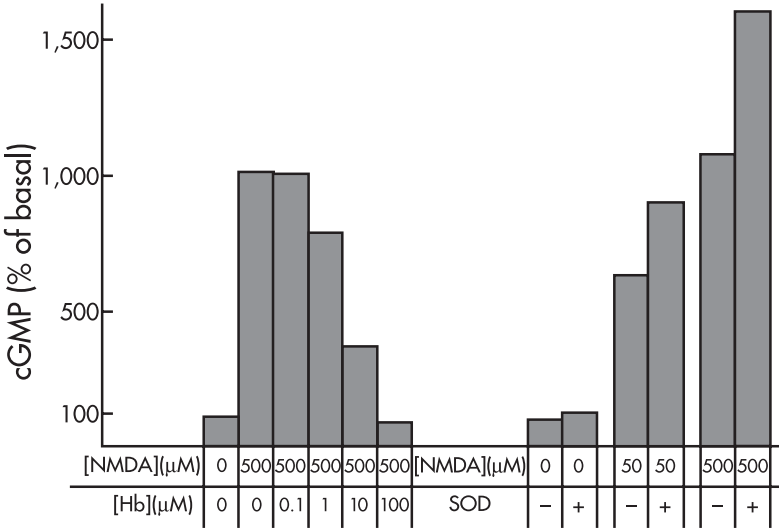


FIGURE 18-5. Inhibition by hemoglobin (Hb) and potentiation by superoxide dismutase (SOD) of NMDA-mediated increase in cGMP in cerebellar slices.

Hemoglobin and superoxide dismutase (100 units/mL) were added 5 min prior to NMDA. Basal level of cGMP was 2.7 pmol/mg of protein. Values are the mean of triplicate determinations with SD <15%. The experiment was repeated twice. +, superoxide dismutase added; -, no superoxide dismutase added.

nescence. In this technique only one sample can be processed at a time, and nitroso derivatives are also detected.

We have obtained definitive evidence that NO mediates the stimulation by glutamate of cGMP levels in the cerebellum. Thus, the relative potencies of glutamate and several derivatives in stimulating the conversion of arginine to citrulline closely parallel their potencies in enhancing cGMP formation. The concentration–response curves for NMDA in elevating cGMP levels and stimulating citrulline formation are superimposable as are such curves for inhibition by MeArg of cGMP and citrulline formation. Inhibition by MeArg of cGMP formation is selectively reversed by arginine. Finally, hemoglobin, which potently complexes NO, inhibits NMDA stimulation of cGMP, and superoxide dismutase, which augments NO levels, enhances the formation of cGMP in response to NMDA.

The relative potencies of NMDA, glutamate, kainate, and quisqualate on [3 H]citrulline and cGMP formation differ from any known excitatory amino acid receptor subtype (20). Since activation of climbing fibers dramatically augments cerebellar cGMP levels, while no known influence of parallel fibers has been described, it seems probable that Purkinje cell cGMP levels are

most prominently regulated by climbing-fiber input. Aspartate is the putative transmitter of climbing fibers (21), suggesting that this unique receptor is selective for aspartate. In the absence of definitive evidence, it might best be designated the excitatory amino acid–cGMP (or EAA–cGMP) receptor. The identical amino acid specificity for [^3H]citrulline and cGMP augmentation indicates that they involve the excitatory amino acid–cGMP receptor and supports a causal link between them.

What is the locus of cGMP in the cerebellum that is regulated by glutamate receptors? Immunohistochemical studies reveal a highly selective and pronounced concentration of cGMP-dependent protein kinase in Purkinje cells (16). Basal levels of cGMP are localized to Purkinje cells, since Purkinje cell-deficient mutant mice demonstrate a 70% depletion of cGMP (22). Immunohistochemical stains for guanylate cyclase localize this enzyme to Purkinje cells (15). Administration of harmaline to rats, which stimulates the firing of climbing fibers and the release of excitatory amino acid neurotransmitters at Purkinje cell synapses, markedly augments cerebellar levels of cGMP (23). The two excitatory inputs to Purkinje cells are climbing fibers and parallel fibers, the latter emanating from granule cells. Glutamate is thought to be the neurotransmitter of these parallel fibers. Parallel fibers can influence Purkinje cells by opening ion channels (24) or stimulating phosphatidyl inositol turnover (25), but it has not been established whether they can regulate cGMP levels. Although a substantial body of evidence suggests Purkinje cells as a site where cGMP levels are regulated, other evidence implies an involvement of granule cells and glia (26). Experiments showing the release of a factor that relaxes smooth muscle and whose properties resemble NO imply the formation of NO by granule cells and stimulation of guanylate cyclase in glia (5).

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CHAPTER 19

Cloned and Expressed Nitric Oxide Synthase Structurally Resembles Cytochrome P-450 Reductase

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Nitric oxide (NO) has recently been discovered to be a messenger for blood vessels and neurons. It accounts for the activity of endothelium-derived relaxing factor (EDRF), which stimulates vasodilatation by releasing NO from the endothelium, which in turn acts on adjacent smooth muscle (1, 2, 3). NO is also responsible for the cytotoxic actions of macrophages (4). In the brain, NO mediates the actions of the excitatory neurotransmitter glutamate in stimulating cyclic GMP concentrations, as inhibition of NO synthase (NOS; EC number 1.14.23) blocks elevations in cGMP (5, 6). Immunohistochemical studies have localized NOS to particular neuronal populations in the brain and periphery (7). Inhibitors of NOS block physiological relaxation of the intestine induced by neuronal stimulation, indicating that NO has the properties of a “neurotransmitter” (8, 9, 10). Thus, NO seems to be a novel

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type of neuronal messenger, in that, unlike conventional neurotransmitters, it is not stored in synaptic vesicles and does not act on typical receptor proteins of synaptic membranes. Its actions in activation of guanylyl cyclase (11) depend on its free-radical character, raising the question of whether other free-radical systems could be biological messengers.

NOS is a highly regulated enzyme. The brain-endothelial enzyme has an absolute requirement for calmodulin (12). NOS requires NADPH (13, 14) and also has tightly bound flavins (D.S.B. and S.H.S., unpublished observations). Brain NOS is stoichiometrically phosphorylated by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin-dependent protein kinase (our unpublished observations). NOS also has a recognition site for arginine, which is converted to NO and citrulline by a novel but so far uncharacterized process which requires molecular oxygen (15).

So that we can find out how NOS catalyzes the transformation of arginine to NO, how this process is regulated by factors, and whether NOS resembles other enzymes that would give rise to free radicals, we have determined its full amino-acid sequence. We report here the cloning and expression of a complementary DNA for brain NOS. The sequence of NOS has striking similarities to that of cytochrome P-450 reductase, another oxidative enzyme which is also unusual in having recognition sites for two flavins and NADPH (16).

Cloning of NOS

NOS protein was purified from rat cerebellum as previously described (12), digested with trypsin, and purified fragments sequenced. Because of the low abundance of NOS protein and messenger RNA, we developed a sensitive two-phased polymerase chain reaction (PCR) cloning strategy which should be useful for cloning other rare mRNA molecules (see legend to Figure 19-1). This yielded a fragment of 599 base pairs (bp) which had the expected amino acids next to each of the primers and was used to screen 10^6 clones of a rat brain cDNA library (Stratagene), and eight independent recombinant phage were isolated. Three of these clones, spanning 5,057 bases, had an open reading frame of 4,287 bases encoding a protein of 1,429 amino acids and of relative molecular mass 160,458 ($M_r \sim 60K$) and incorporates all 21 peptides that had been sequenced previously. The start codon was assigned to the first methionine in the open reading frame at nucleotide 349 because the neighboring bases, ATACC(ATG)G, conform well to the consensus for initiation of translation (17).

Functional Expression of NOS

The full-length coding cDNA was inserted into an expression vector using a cytomegalovirus promoter which was transfected into human kidney 293

cells. Coomassie-blue staining of cell extracts collected 3 days after transfection reveals one band in transfected cells not present in untransfected cells and having an M_r of 160K, corresponding to the size of purified NOS (12; Figure 19–2). NOS protein corresponds to about 1–2% of total protein in the transfected cells, whereas negligible amounts are present in the untransfected cells. Western blot analysis using an antiserum that is highly selective for NOS (7) reveals a single 160K band in the transfected cells which is not present in the untransfected cells and which corresponds to NOS in cerebellar extracts (Figure 19–2). The immunoreactive band identified from 30 μ g of transfected cells is more intensely labeled than is the band from 200 μ g of cerebellar tissue, consistent with a 10-fold enrichment of NOS in the transfected cells over the cerebellum.

NOS catalytic activity in transfected cells was assayed in three ways, measuring 1) the conversion of [3 H]arginine to [3 H]citrulline; 2) the production of nitrite from unlabeled arginine; and 3) the enhancement of endogenous guanylyl cyclase activity in response to newly synthesized NO (Figure 19–3). With all three assays there is negligible NOS activity in untransfected cells, whereas there is great activity in transfected cells. This activity is about 10 times greater than that in cerebellar extracts, the richest known mammalian source of NOS activity of the brain-endothelial type. The NOS activity is about 1% of that of purified brain NOS, suggesting that it accounts for 1% of the protein in the transfected cells, which fits with the estimate based on Coomassie-staining of extracts from these cells. NOS activity of the transfected cells corresponds to the properties of the enzyme characterized in the brain (12). Enzyme activity is virtually lost in the absence of NADPH or calcium and is inhibited by trifluoperazine, a calmodulin antagonist; elevations in guanylyl cyclase activity are blocked by hemoglobin, which binds NO (Figure 19–3).

NOS mRNA Expression

To evaluate the expression of the NOS gene, we analyzed various tissues and brain regions by Northern blotting (Figure 19–4). There is a prominent 10.5-kb band in total cerebellar RNA. Because the coding sequence for NOS is only about 4.3 kb, around half of its mRNA is not translated. We do not detect NOS mRNA in kidney, liver, skeletal muscle, stomach, or heart. In the brain, NOS mRNA levels are highest in the cerebellum, followed in descending order by olfactory bulb, colliculi, hippocampus, and cerebral cortex. The relative amounts of mRNA in these brain regions correspond closely to the relative amounts of NOS protein and catalytic activity (7).

We localized NOS mRNA *in situ* at a microscopic level using hybridization (Figure 19–5a). It is most prominent in the cerebellum, olfactory bulb,

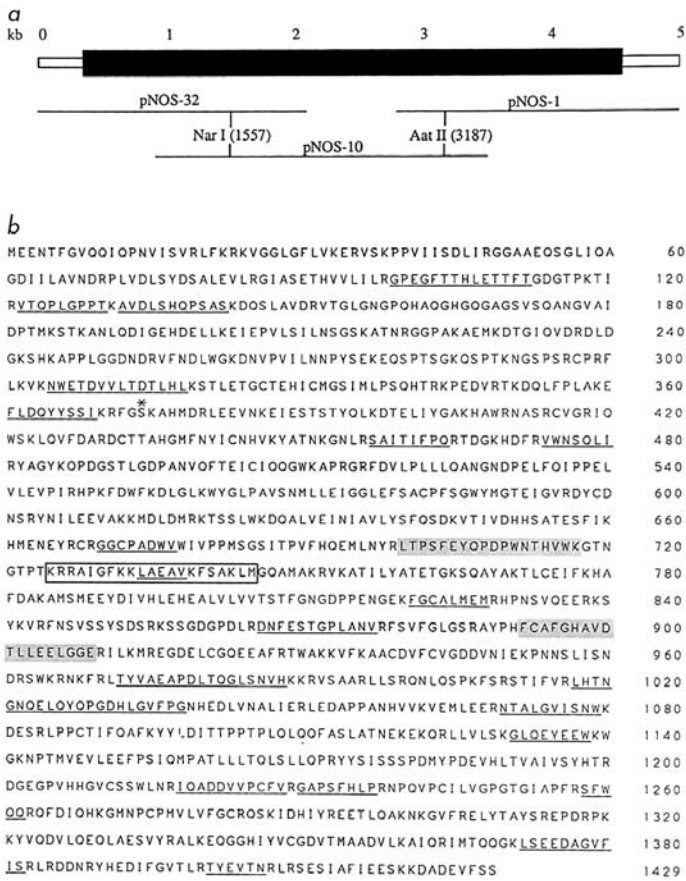


FIGURE 19-1. Amino-acid sequence of NOS (single-letter amino-acid notation).

(a) Diagram of the overlapping partial cDNAs encoding NOS and the restriction sites used for their ligation. The translated sequence is indicated by a solid bar. (b) Predicted amino-acid sequence of NOS. The tryptic peptides sequenced from the purified protein are underlined. The two peptides used for the initial PCR cloning are shaded. Asterisk indicates the consensus sequence for phosphorylation by protein kinase A; the predicted binding site for calmodulin is boxed.

Methods. The two-round PCR cloning strategy is as follows. First, PCR primers were designed on the basis of the amino-acid sequences of the two largest peptides sequenced, 17 and 18 amino acids, respectively. For each of these peptides, a pair of fully degenerate oligonucleotides were constructed to match the six amino acids at the N and C termini, and these were used to amplify the short intervening sequences between the primers by PCR at 42°C annealing temperature on first-strand complementary DNA derived from rat brain RNA. After amplification, the single prominent PCR product of the expected size from each reaction was excised from an agarose gel, cloned,

FIGURE 19-1. Amino-acid sequence of NOS (single-letter amino-acid notation) (continued).

and sequenced to reveal the nucleotides encoding the five or six central amino acids of each peptide. Nondegenerate oligonucleotides corresponding to the sequences determined from these central regions were used in a second round of PCR done at a much higher annealing temperature (63°C) on first-strand rat brain cDNA. Two pairs of oligonucleotides were synthesized as the relative order of the two peptides in the protein sequence of NOS were unknown. One pair of oligonucleotides gave no PCR product, whereas the other produced a single 599-bp product. This technique was used for plaque hybridization to isolate cDNA clones from a rat brain library. The PCR product was random-prime labeled (1×10^6 c.p.m. ^{32}P ng $^{-1}$), hybridized to replicate filters overnight, and the filters were washed with high stringency (0.1×SSC medium, 0.1% SDS, 65°C). Overlapping clones were sequenced by the dideoxy chain termination method using a commercial kit, Sequenase (USB). All PCR primers included nine-bp sequences at the 5' end which allowed subcloning into *Eco*R1 or *Bam*H1 sites. The first round of PCR (42°C annealing temperature, 36 cycles) was performed with the following primers: peptide 1, CCGGAATTCCTNACNCCNTCNTT(T/C)GA and GCCGGATCC(C/T)TTNAC(A/G)TGNGT(A/G)TTCCA; peptide 2, CCGGAATTC-TT(T/C)TG(T/C)GCNTT(T/C)GGNCA, and GCCGGATCC(T/C)TCNCCNCCN-AG(T/C)TC(T/C)TC. Primers used for the second round of PCR (63°C annealing temperature, 36 cycles) were CCGGAATTCGAATACCAGCCTGATCCATGGAA and GCCGGATCCTCCAGGAGGGTGTCCACCGCATG. The start codon is at position 349 and the stop at 4,636.

and pedunculo pontine tegmental nucleus, structures which are seen to be highly enriched in NOS when using immunohistochemistry (7; and unpublished observations). In the cerebellum, NOS mRNA is most enriched in the granule cell layer, and also enriched in the main olfactory bulb, dentate gyrus of the hippocampus, supraoptic nucleus, and superior and inferior colliculi. The white dots in the cerebral cortex, caudate putamen, and basal forebrain represent isolated NOS-containing neurons, the distribution of which resembles that seen by immunohistochemistry (18). High-power micrographs of the caudate putamen reveal NOS mRNA localized to large aspiny neurons (Figure 19-5c), which are also selectively enriched in NADPH diaphorase staining (Figure 19-5b).

Primary Structure of NOS

The NOS amino-acid sequence has a number of recognition sites needed for enzyme's function (Figures 19-1 and 19-6). There is a basic amphipathic α helix calmodulin-binding consensus sequence (19) at position 725-745, consistent with its requirement of calmodulin (12). A cAMP-dependent protein kinase phosphorylation consensus sequence (20), KRFGS, is at position 473, consistent with a serine phosphorylation by cAMP-dependent protein kinase and fit-

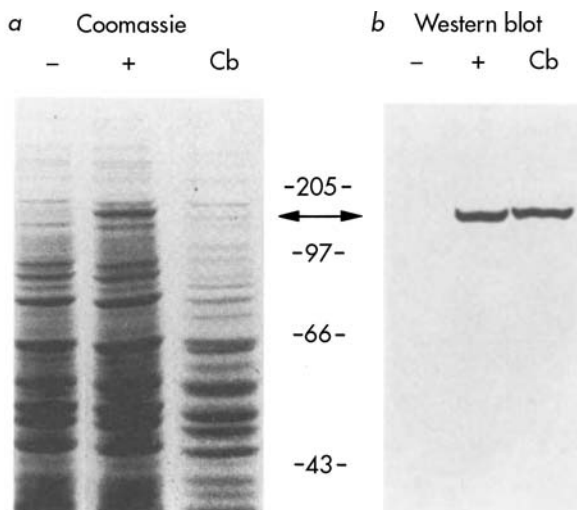


FIGURE 19-2. Expression of NOS protein (a), Coomassie-blue staining, and (b) Western blot analysis of soluble extracts from rat cerebellum (Cb) and human kidney cells following transfection with control expression vector (-) or NOS vector (+).

Positions of M_r markers are shown ($M_r \times 10^{-3}$); double-headed arrow at 160K indicates the position of NOS protein.

Methods. cDNA clones spanning the full open reading frame for NOS were combined as shown in Figure 19-1a, excised from bluescript with *Cla*I and *Xba*I, and ligated into the expression vector p-CIS-2 (Genentech). Human 293 kidney cells were transfected with calcium phosphate as described (42), cells were collected 3 days after transfection, homogenized in buffer containing 1 mM EDTA, and centrifuged at 100,000g for 60 min. Soluble extracts from transfected kidney cells (30 μ g per lane) or rat cerebellum (30 μ g for Coomassie-blue staining, 200 μ g for Western blot) were separated on a 7.5% SDS polyacrylamide gel, then either stained with Coomassie blue or transferred to nitrocellulose, probed with anti-NOS antibody (7) overnight at 1:5,000 dilution, and the bands visualized with an alkaline phosphatase-linked secondary antibody.

ting with the observed phosphorylation by this enzyme. There are no consensus sequences for phosphorylation by protein kinase C or calcium/calmodulin protein kinase which are selective enough to be identified. An NADPH-binding domain makes up the area from amino acids 1,204 to 1,429 (21). Well-defined sites representing the point of contact with the ribose and adenine rings are at amino acids 1,245-1,263 and 1,343-1,358, respectively, and are closely homologous to similar sites in cytochrome P-450 reductase, sulphite reductase, and ferredoxin NADP⁺ reductase (22-26) (Figure 19-6A).

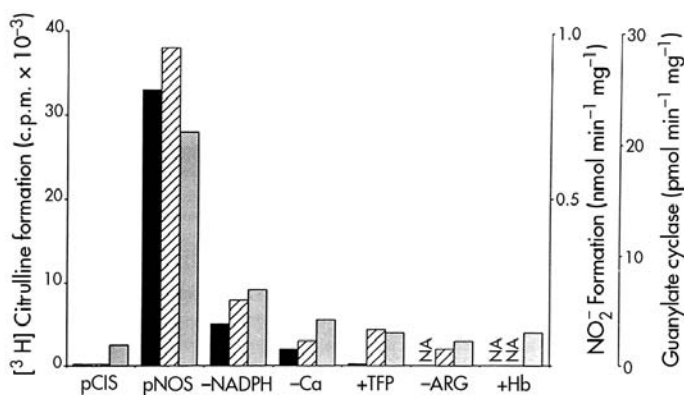


FIGURE 19-3. Functional expression of NOS enzyme activity.

NOS activity was measured in control transfected kidney cells (pCIS) and in cells transfected with NOS expression vector (pNOS). Enzyme activity was assayed in three ways: 1) conversion of [³H]arginine to [³H]citrulline (solid bars); 2) formation of NO₂⁻ from unlabeled arginine (striped bars); and 3) activation of endogenous soluble guanylyl cyclase by arginine (dotted bars). For the first two sets of bars, enzyme activity was measured with complete reaction mixtures and found only in NOS-transfected cells. The final five sets all use NOS-transfected tissue. Data are means of triplicate determinations from a representative experiment.

Methods. Kidney cells were transfected, soluble extracts were prepared as described in Figure 19-2 and dialyzed for 12 h to remove most endogenous arginine and NADPH. [³H]Citrulline formation from [³H]arginine and NO₂⁻ production from unlabeled arginine were monitored as described (5). Guanylyl cyclase activity was assayed by monitoring cyclization of [α -³²P]GTP to [³²P]cGMP as described (38). The standard reaction mixture was 20 mM Tris-HCl (pH 7.4), 1 μ M free Ca²⁺, 1 mM NADPH. For [³H]citrulline assays, 1 μ M [³H]arginine (100,000 c.p.m.) was added; for nitrate assays, 1 mM arginine was added; for guanylyl cyclase assays, 1 mM arginine, 1 mM cGMP, 0.1 mM [α -³²P]GTP, 5 mM Mg²⁺, 1 mM isobutylmethyl xanthine, and 50 units mL⁻¹ superoxide dismutase were added. TFP, 100 μ M trifluoperazine; Hb, 10 μ M hemoglobin; NA, not applicable.

Enzymes requiring NADPH typically use other cofactors as well. Tetrahydrobiopterin enhances the activity of macrophage NOS (27, 28) and stimulates porcine brain NOS (29). The brain NOS sequence has consensus binding sites for the flavins FMN and FAD (Figure 19-5). There are distinct binding sites for the pyrophosphate and isoalloxazine moieties at the FAD region (21). These flavin-binding sites are specifically conserved in a family of electron transfer proteins (21). Using fluorescence spectroscopy we have demonstrated the stoichiometric occurrence of tightly bound FMN and FAD in purified rat brain NOS (unpublished results).

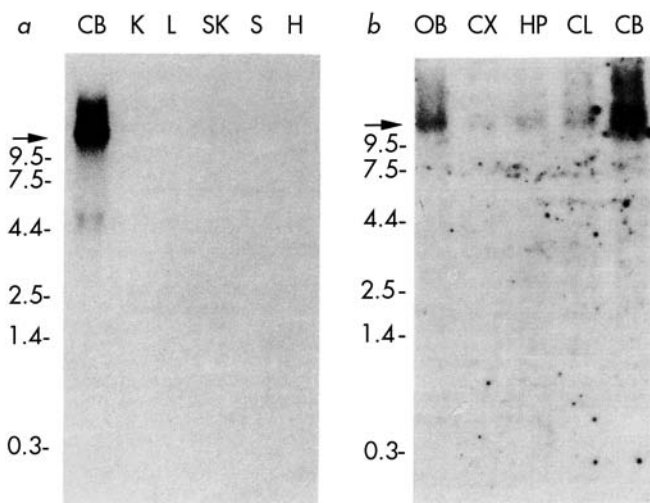


FIGURE 19-4. NOS mRNA expression in brain and peripheral tissues.

(a), Northern blot analysis of total RNA (20 μ g) from cerebellum (CB), kidney (K), liver (L), skeletal muscle (SK), stomach (S), and heart (H). The arrow indicates the NOS message present only in cerebellum at 10.5 kb. (b), Regional distribution of NOS mRNA in brain reveals highest levels in cerebellum (CB), followed by olfactory bulb (OB), colliculi (CL), hippocampus (HP), and cerebral cortex (CX). Positions of M_r (K) markers are shown.

Methods. RNA was purified from rat tissues by tissue lysis in guanidinium isothiocyanate followed by centrifugation through CsCl (44). The RNA samples were separated on a formaldehyde agarose gel, probed with a random prime-labeled (3×10^6 c.p.m. ^{32}P ng $^{-1}$; 10^8 c.p.m. per filter) full-coding region cDNA (bases 1–5,057), washed at high stringency (0.5 \times SSC medium, 0.1% SDS, 65°C), and exposed to X-ray film for 42 days.

The amino-acid sequence of NOS was compared with that of all sequenced proteins in GenBank using a FASTA program. The closest homology is to rat cytochrome P-450 reductase (Figure 19-6B). The two proteins are similar at the C-terminal half of NOS which, over 641 amino acids, has 36% identity and 58% close homology to cytochrome P-450 reductase. The only other protein with substantial homology to NOS is sulphite reductase which has 32% identity and 49% close homology to NOS over the C-terminal 641 amino acids of NOS. NOS, cytochrome P-450 reductase, and sulphite reductase are a unique class of electron transferase enzymes characterized by binding sites for NADPH, FMN, and FAD in the same polypeptide. For the two reductases, the flavins have been shown to make up a transport chain with electrons shuttling between the isoalloxazine rings. Specific sequences in cytochrome P-450 reductase might be responsible for coupling the FAD

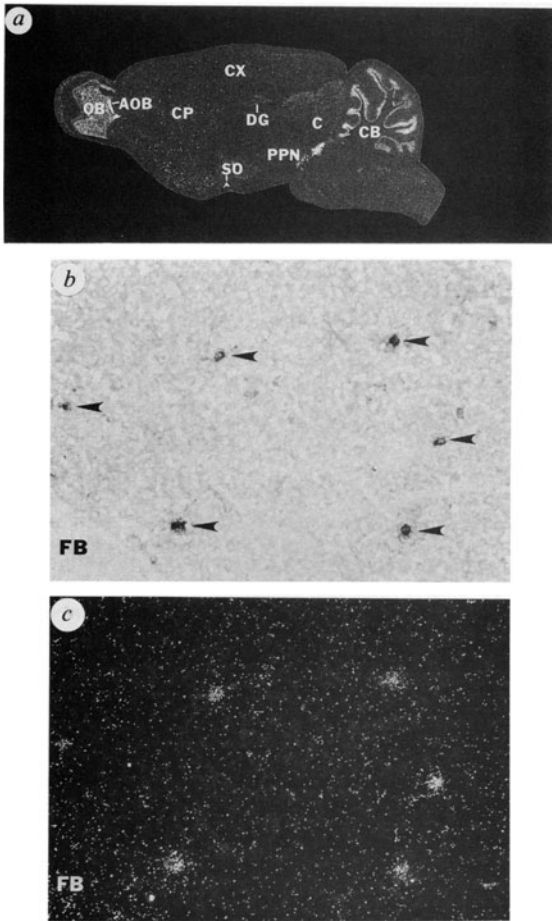


FIGURE 19-5. Localization of NOS mRNA in brain.

(a) *In situ* hybridization to NOS mRNA in a sagittal section of rat brain in darkfield shows densest message levels in granule cell layer of cerebellum (CB), accessory olfactory bulb (AOB), and pedunculopontine tegmental nucleus (PPN). High densities of grains are also apparent in the main olfactory bulb (OB), dentate gyrus (DG), supraoptic nucleus (SO), and superior and inferior colliculi (C), white dots scattered throughout the cerebral cortex (CX), caudate putamen (CP), and basal forebrain represent isolated NOS-containing neurons in these regions. (b) High-power lightfield micrograph showing coincidence of NADPH diaphorase staining of aspiny neurons (arrowheads) in caudate putamen with (c), NOS mRNA *in situ* on the same section viewed under darkfield. FB, fiber bundle.

Methods. *In situ* hybridization was done as described (45) with a pool of three end-labeled 45-base oligonucleotides complementary to bases 211–266, 795–840, and 4,712–4,757. For the colocalization, NADPH diaphorase staining was done immediately before *in situ* hybridization as described (46).

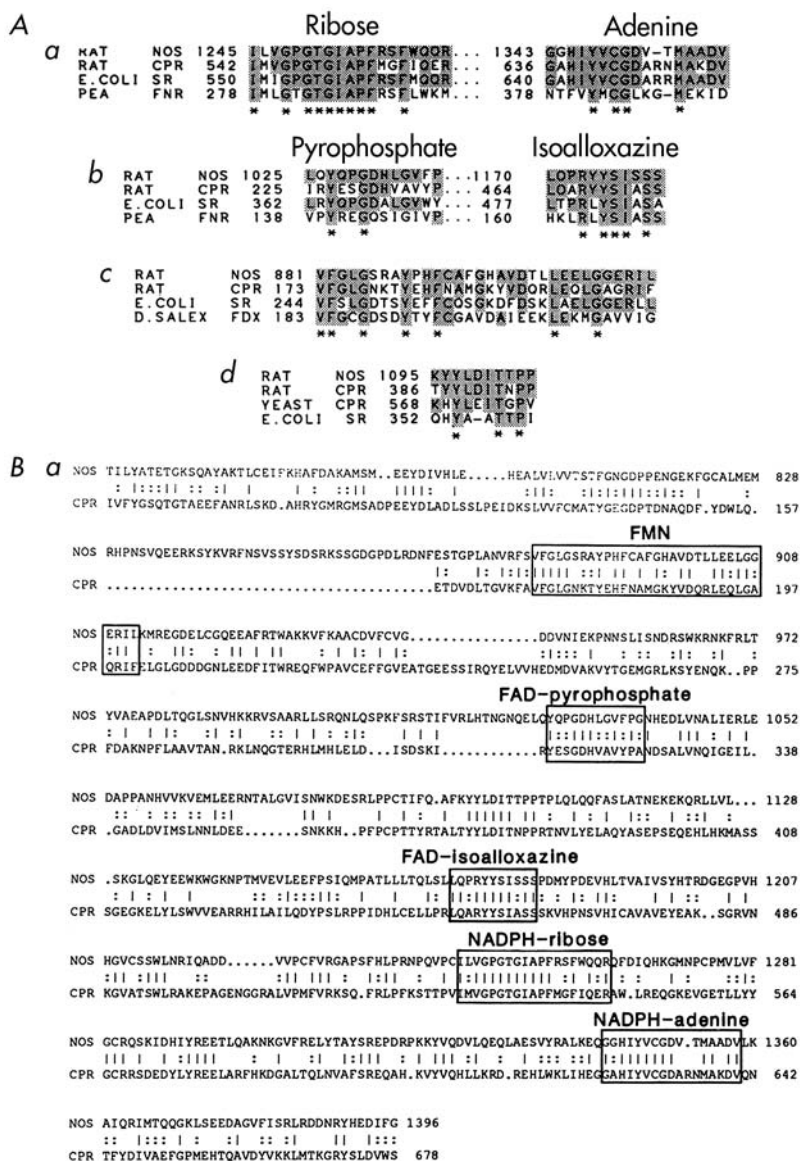


FIGURE 19-6. (A) Sequence alignment of NOS with cofactor binding regions in other electron transferase enzymes.

High degrees of sequence identity correspond to regions previously identified in other proteins to be responsible for FMN binding (c), FAD binding (b), and NADPH binding (a) (CPR, cytochrome P-450 reductase; SR, sulphite reductase; FDX, flavodoxin; FNR, ferredoxin NADP⁺ reductase; and D. Salex, *Desulfovibrio salexigens*). (d) A region of high homology present only in proteins known to bind both FAD and FMN may be involved in facilitating electron transfer between the flavins. Amino acids identical to NOS are shaded; asterisks indicate consensus amino acids present in all four sequences.

(B) Sequence alignment of NOS with cytochrome P-450 reductase (CPR).

(a) The amino-acid alignment of NOS with cytochrome P-450 reductase demonstrates 36% sequence identity and 58% homology across the final 641 amino acids of NOS. Identical amino acids are indicated by a solid line and homologous substitutions by two dots. (b) A schematic model showing the spatial relationships of the cofactor recognition sites within NOS and CPR. The predicted sites for calmodulin binding and protein kinase A phosphorylation within the NOS sequence and the transmembrane domain (TMD) in the CPR sequence are noted.

Methods. Sequences were aligned with a FASTA program. Conservative substitutions were defined as (1) C; (2) S, T, P, A, G; (3) N, D, E, Q; (4) H, R, K; (5) M, I, L, V; (6) F, Y, W.

and FMN regions (21). NOS has a similar sequence (Figure 19-6A) and thus electron transfer between the flavins probably has a role in the mechanism of NO synthesis. The N-terminal half of NOS has no significant homology to any known protein.

NOS Gene Family

Clearly there are several distinct NOS enzymes. The brain and endothelium seem to use the same or similar calmodulin-dependent enzyme, brain NOS (bNOS), as both are recognized by a specific antiserum to bNOS (7). The macrophage (30) and neutrophil (31) must each have distinct enzymes as neither requires calmodulin for activity and neither is labeled by antiserum to bNOS (7). We have recently identified a gene product highly homologous to bNOS that is expressed selectively in activated macrophages and is probably the macrophage enzyme, macNOS (31a).

Discussion

Because of the selective close similarity of amino-acid sequence between cytochrome P-450 reductase and NOS, we wondered whether the two enzymes have analogous functions. Cytochrome P-450 reductase provides the elec-

trons for the activities of the microsomal P-450 enzymes (32). Histochemical analysis in the brain shows selective neuronal localizations for a number of P-450 enzymes (33, 34), as well as for cytochrome P-450 reductase in discrete populations of catecholamine neurons (35). Besides these catecholamine neurons, cytochrome P-450 reductase also occurs in a number of other selected neuronal populations such as Purkinje cells of the cerebellum (M. Fotuhi, T. Dawson, S.H.S., unpublished results). In the liver and certain other peripheral tissues, the P-450 enzymes are primarily associated with drug metabolism. This would seem unlikely for P-450 enzymes and cytochrome P-450 reductase in the brain, especially in light of the localization of these enzymes to very discrete neuronal populations in the brain. Conceivably, oxidative products formed by the P-450 system have a messenger role in discrete neuronal populations similar to that of NO.

The reasons for selectively high production of NO by very particular populations of neurons have been obscure. Although NO mediates the effects of glutamate in elevating cGMP amounts in the cerebellum (5, 6), the localization of NOS in brain does not resemble that of guanylyl cyclase mapped by immunohistochemistry (36), suggesting that NO has multiple functions in the brain. We find the mRNA for NOS to be colocalized with neurons selectively enriched in NADPH diaphorase, complementing our findings by immunohistochemistry, which demonstrate that NOS activity fully accounts for NADPH diaphorase activity (18, 37). NADPH diaphorase has been characterized by histochemical staining to have an oxidative activity dependent on NADPH but not NADH (38). NADPH diaphorase neurons are selectively resistant to ischemic destruction and survive effects of excitatory neurotoxins as well as the degenerative process of Huntington's disease (39, 40, 41, 42). Thus, one function of NO may be to protect neurons from ischemic and neurotoxic insults. NOS inhibitors selectively prevent glutamatergic neurotoxicity mediated by *N*-methyl-D-aspartate receptors in primary brain cultures, indicating that NO secreted by NOS neurons may kill adjacent neurons (43).

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CHAPTER 20

Behavioral Abnormalities in Male Mice Lacking Neuronal Nitric Oxide Synthase

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In addition to its role in blood vessel (1, 2) and macrophage (3, 4) function, nitric oxide (NO) is a neurotransmitter (5) found in high densities in emotion-regulating brain regions (6, 7, 8). Mice with targeted disruption of neuronal NO synthase (nNOS) display grossly normal appearance, locomotor activity, breeding (9), long-term potentiation (10), and long-term depression (11). The nNOS⁻ mice are resistant to neural stroke damage following middle cerebral artery ligation (12). Although CO₂-induced cerebral vasodilatation in wild-type mice is NO-dependent, in nNOS⁻ mice this vasodilation is unaffected by NOS inhibitors (13). Establishing a behavioral role for NO has, until now, not

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been feasible, as NOS inhibitor drugs can only be administered acutely and because their pronounced effects on blood pressure and other body functions obfuscate behavioral interpretations. We now report a large increase in aggressive behavior and excess, inappropriate sexual behavior in nNOS⁻ mice.

In establishing our breeding colony of nNOS⁻ mice, we housed groups of five nNOS⁻ male mice together in cages and upon routine morning examinations often discovered one or two dead mice in each cage. Initial direct observations indicate that the male nNOS⁻ mice engage in chronic aggressive behavior, not apparent among nNOS⁻ female mice or wild-type male or female mice housed together (Figure 20-1).

To examine this behavior in greater detail, we conducted studies of intermale aggression in an intruder-resident model (14). The following aggressive behaviors were scored: offensive attack, biting, wrestling, and chasing. Tail rattling was not recorded as an aggressive behavior. Submissive postures were also recorded. Submission was operationally defined as rolling onto the back with the paws extended. When wild-type mice are introduced to a cage, the latency to the first aggressive attack by the resident mice is the same for wild-type and nNOS⁻ residents (Figure 20-2). However, the nNOS⁻ residents display 3-4 times more aggressive encounters than wild-type residents. Moreover, in these encounters attacks are initiated by the nNOS⁻ residents in 87% of their encounters with the wild-type intruders, nearly six times higher than the proportion of attacks initiated by wild-type residents.

In another model we observed groups of four wild-type or nNOS⁻ males together in an aquarium (Figure 20-3). In this model the latency to first attack is less than 1 min for the nNOS⁻ animals, about one-fifth the latency for wild-type mice. The nNOS⁻ mice display almost twice as many attacks as wild-type animals during a 15 min observation period. It is likely that the disparity between the nNOS⁻ and wild-type mice would be greater with a longer duration, but we stopped the encounters after 15 min to prevent serious wounding. The duration of aggressive encounters is also greater in nNOS⁻ than wild-type mice. The most dramatic difference between the two groups involves the number of submissive postures. nNOS⁻ mice display submissive postures only one-tenth as frequently as wild-type mice.

Inappropriate aggressiveness has only been observed in male nNOS⁻ mice. In multiple experiments female nNOS⁻ mice have not exhibited aggressive behavior when challenged by an intruder female. Furthermore, female nNOS⁻ mice grouped 4 per cage also do not display any aggressive behaviors.

Our initial behavioral observations suggested that the persistence of excessive behaviors seen in aggressive encounters extends to reproductive interactions. In our efforts to obtain timed pregnant females, we paired nNOS⁻ males with nNOS⁻ females at varying stages of estrus. The males display excessive and inappropriate mounting behavior associated with

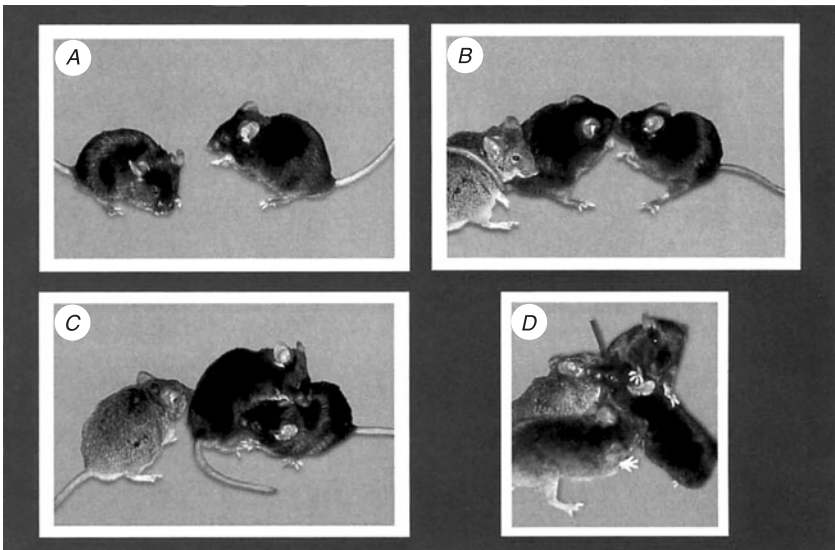


FIGURE 20-1. Photographs of $n\text{NOS}^-$ male mice engaged in an aggressive encounter.

In (A), the mice are preparing to engage. Both black $n\text{NOS}^-$ mice in (B) are in the classic boxing stance while a brown $n\text{NOS}^-$ mouse looks on. In (C), the two black $n\text{NOS}^-$ mice are fighting, and neither mouse has displayed a submissive posture. In (D), the two brown $n\text{NOS}^-$ mice join in the aggressive encounter. These photographs were obtained by simultaneously introducing four $n\text{NOS}^-$ mice into a neutral arena in the experimental model used in Figure 20-3. The photographed interactions commenced about 10 min after the mice were placed in the aquarium. The sequence of depicted events occurred over a 2 min period. $n\text{NOS}$ null mice were obtained from a breeding colony established at Johns Hopkins University using animals previously produced by homologous recombination (9). All animals were screened for homozygosity of the $n\text{NOS}$ null mutation by Southern blot analysis. Wild-type animals consisted of both age-matched C57B6J and SvEv 129 strains to control for possible strain effects because of the $n\text{NOS}$ null mice genetic background. Additional controls consisted of age-matched heterozygous $n\text{NOS}^{+/-}$ littermates who displayed normal behavior when males were paired together, similar to wild-type mice. Animals were 4–6 months of age at the onset of testing (sexually mature), and were maintained individually in LD 16:8 photoperiods (light on 7:00 EST) at $20 \pm 2^\circ\text{C}$ temperatures and relative humidity of $50 \pm 5\%$ throughout the study. Food (Prolab 1000; Syracuse, NY) and tap water were available ad libitum. All statistical comparisons involved one-way analyses of variance (ANOVA) except where otherwise noted. Pair-wise comparisons were accomplished with Student's t -tests. Mean differences were considered statistically significant if $P < 0.05$. Aggression tests were conducted as described previously (14).

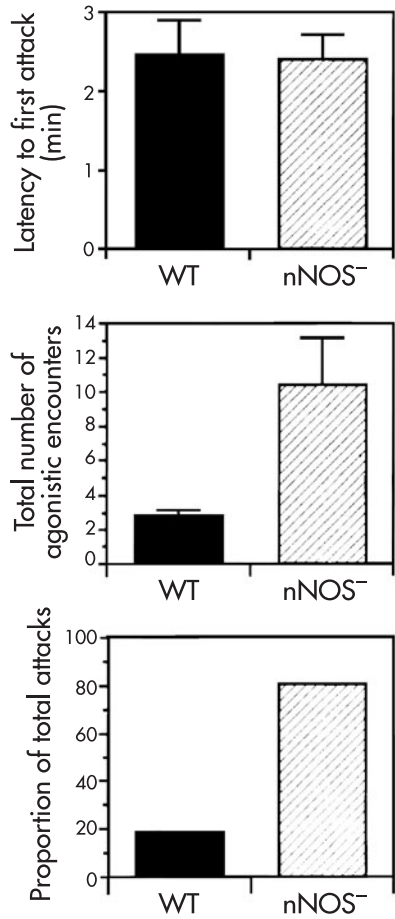


FIGURE 20-2. Defensive aggressive behavior in nNOS⁻ and wild-type mice.

These tests were modified from the procedures described previously to assess aggression in genetic knockout mice (15, 16). A wild-type intruder was introduced into the home cage of either an nNOS⁻ or wild-type adult male mouse ($n=8$), and the defensive aggressive behaviors were recorded. The latency to first aggressive encounter, the number of agonistic encounters, and the proportion of aggressive behaviors initiated by the resident male during the 5-min tests were recorded. Aggression tests were conducted each day for three consecutive days between 15:00 and 17:00, and a unique pairing was made for each test. The data (mean \pm SEM) were combined from the three tests and plotted in this figure. All behavioral tests were scored blindly.

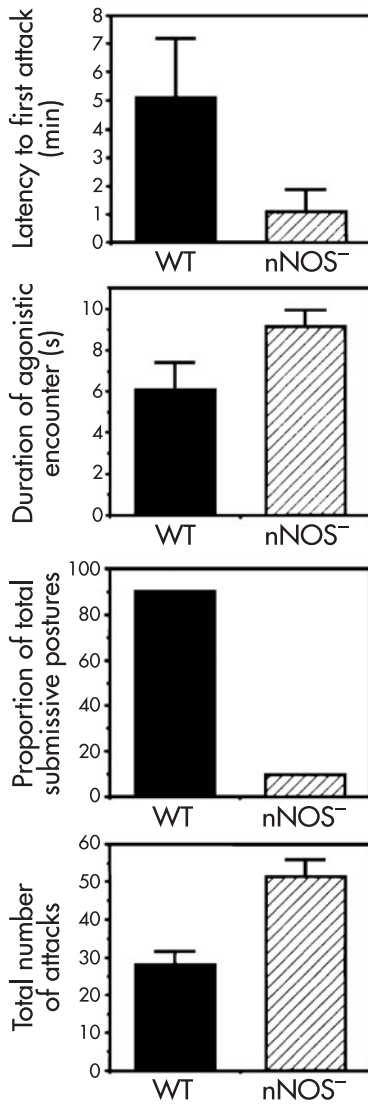


FIGURE 20-3. Offensive aggressive behavior among grouped nNOS⁻ and wild-type male mice in a neutral area.

Four adult male wild-type or four nNOS⁻ ($n=16$) mice were simultaneously introduced into a clear glass aquarium ($38.5 \times 26.5 \times 30.7$ cm). The latency to first aggressive encounter, the duration of each agonistic encounter, the proportion of submissive postures, and the total number of agonistic encounters during the 15 min test were recorded for the entire group of four mice. Aggression tests were conducted on two consecutive days between 11:00 and 13:00, and the data (mean \pm SEM) were combined for this figure. All behavioral tests were scored blindly.

substantial vocal protestations by the females. To quantify this behavior we conducted studies in which anestrous females were paired with wild-type or nNOS⁻ males commencing at 10:00. Animals were observed for 8 h and their behaviors were recorded by videotape and scored blindly. Two “live” observers, also uninformed about the phenotype of the mice, scored the behavioral interaction during the first 15 min of each hour (Figure 20–4). During the initial 15 min observation period wild-type and nNOS⁻ males mount females to the same extent, reflecting the normal male response to a new female. This behavior diminishes rapidly in wild types so that by 1 h after introduction into the cage the number of mounts by wild-type males decreases 50%. By contrast, only a modest, nonsignificant decrease in the number of mounts occurs for the nNOS⁻ animals. By 7 and 8 h following introduction to the cage, wild-type mouse encounters have decreased to levels only one-sixth those of the initial period. At these times the number of mounts by nNOS⁻ animals continues to be 2–3 times greater than values for wild-type animals.

Both in studies of aggression and of sexual behavior the nNOS⁻ animals display a marked increase in inappropriate aggressive and sexual behavior reflected in persistent fighting and mounting behavior despite obvious signals of surrender or disinterest, respectively, by their test partners. Generally, both aggression and sexual behavior are enhanced by elevated testosterone levels. Accordingly, we monitored blood testosterone levels in wild-type and nNOS⁻ males 2 weeks before any behavioral testing as well as 1 h after completion of the aggression tests. Blood plasma testosterone levels do not differ between the nNOS⁻ and the wild-type males at either time point and there is no significant difference in the levels before and after the behavioral study.

Is it possible that sensory or motor abnormalities in the nNOS⁻ animals account for the apparent increase in inappropriate aggressive and sexual behavior? For instance, perhaps the nNOS⁻ animals fail to recognize social inhibitory stimuli, such as an odor emitted by a nonestrous female. Accordingly, we monitored olfactory behavior by assessing the ability of mice to find a cookie hidden beneath the cage bedding. Latency to discover the hidden cookies does not differ between wild-type and nNOS⁻ males and females. Might our behavioral observations be influenced by differences in strength and agility between wild-type and nNOS⁻ animals? We monitored the ability of mice to turn around in a blind alley, to walk across a suspended pole, and to hang from a pole (Figure 20–4 legend). nNOS⁻ males and females do not display any decrease in performance in these measures. Conceivably the nNOS⁻ animals are frightened, leading to increased fighting behavior. We monitored behavioral anxiety in our open field test. We observed no difference between nNOS⁻ and wild-type males and females.

Mice with targeted disruption of genes for calcium/calmodulin kinase II (15) and serotonin receptor 5-HT_{1B} (16) also display enhanced inter-male

aggression. Augmented aggression is not likely to be a general feature of animals with targeted disruption of any gene, as animals with deletion of genes for estrogen receptors exhibit reduced aggressive behavior (17). In some instances (15) mice with gene deletions display major anatomical and physiological abnormalities and appear "sick," which can complicate interpretations of discrete behavioral deficits. For instance, mice with deleted monoamine oxidase A display increased inter-male aggression but also tremble, have difficulty in righting themselves, are fearful and blind (21). No neuroanatomical or physiological disturbances have been observed in the nNOS mice whose behavior superficially appears normal (9).

Though NOS has been implicated in development of cultured PC12 cells (18), iNOS [inducible NOS] rather than nNOS was primarily involved. If nNOS deletion markedly altered neuronal development, one would anticipate substantial neuroanatomical and gross psychomotor abnormalities in nNOS⁻ mice. However, detailed neuroanatomical studies (9) and a thorough sensorimotor evaluation have not detected abnormalities. Also, synaptic plasticity has been evaluated in the nNOS⁻ mice revealing normal long-term potentiation in hippocampal slices (10) and normal long-term depression in cerebellar cultures (11). Accordingly, it is highly probable that the behavioral abnormalities we have observed are direct, selective consequences of the loss of nNOS and not secondary to global physiological disruptions. Though mating and aggression are largely centrally mediated, a role for peripheral and indirect influences such as the dilated stomachs of nNOS⁻ mice cannot be completely excluded (9). As drug-induced serotonin depletion augments aggressive and sexual activity (22, 23) and NO neurons contact serotonin cells (5), the nNOS⁻ mice behavioral abnormalities might involve serotonin.

Our findings provide the first evidence for a behavioral role of central nNOS neurons, which presumably participate in sexual and aggressive behavior. Studies administering NOS inhibitors have implicated NO in alcohol consumption (19) and feeding behavior (20). However, NOS inhibitors such as L-nitroarginine influence macrophage and endothelial NOS as well as nNOS and can affect other biological systems that use arginine. The use of mice with gene deletions overcomes such problems of nonspecificity.

Though direct comparisons are not feasible, the sexual and aggressive aberrations of nNOS⁻ mice seem more pronounced than those reported with deletion of other genes. Accordingly, NO may be a major mediator of sexual and aggressive behaviors, relevant for studies of their biological determination in humans as well as mice. Studies of monoamine oxidase A indicate the relevance to humans of aggressive behavior in mice. Mice with deletions of the gene for this enzyme display excessive aggression (21), and humans with low levels of the enzyme are also hyperaggressive (24).

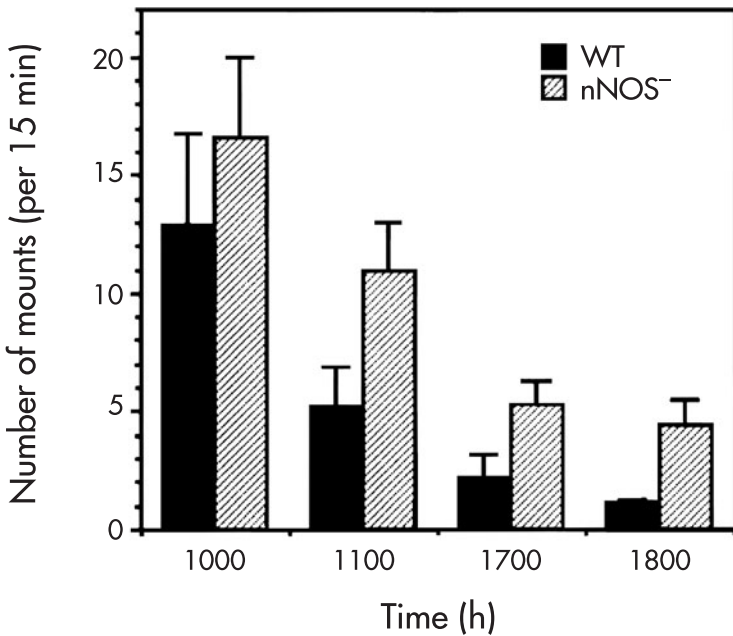


FIGURE 20-4. Sexual behavior of nNOS⁻ and wild-type mice.

The mean (\pm SEM) number of mounts (per 15 min observation period) were monitored for nNOS⁻ ($n=8$) or wild-type ($n=8$) male mice when paired with a non-estrous female in a neutral arena. Males were initially placed in a clear aquarium (38.5 \times 26.5 \times 30.7 cm) for a 15 min acclimatization period. After this a non-estrous wild-type female was introduced into the mating arena. Animals remained paired for 8 h from 10:00 to 18:00. The number of mounts and aggressive behaviors were recorded by two observers, who were uninformed about the genotype of the mice, during a 15 min observation period at the start of each hour. The behavioral tests were also videotaped continuously for subsequent confirmation of the live scoring. A mount was operationally defined as the male assuming the copulatory position, but failing to achieve intromission. Intromission was defined as the male's penis entering the vagina in association with thrusting behavior. No animals displayed an intromission. There was only one observed incidence of aggressive behavior, and it was performed by a nNOS⁻ mouse. Mean (\pm SEM) blood plasma testosterone levels of nNOS⁻ and wild-type male mice before the start of the behavioral testing were 1.95 ± 0.22 and 2.14 ± 0.14 ng mL⁻¹, respectively ($P > 0.05$); at the end of the experiment, mean (\pm s.e.m.) testosterone values of nNOS⁻ and wild-type males were 1.86 ± 0.12 and 1.98 ± 0.01 ng mL⁻¹, respectively ($P > 0.05$). The basal blood sample was obtained 10 days after the mice arrived in the behavioral laboratory and 2 weeks before the onset of behavioral testing. Another sample was obtained 1 h after the behavioral tests were completed. Blood samples were obtained from the retro-orbital sinus of mice that were lightly anesthetized with methoxyflurane vapors (Metofane; Pitman-Moore, Mundelein, IL). Blood was centrifuged at 3,500 r.p.m. for 1 h at 4°C; plasma

FIGURE 20-4. Sexual behavior of nNOS⁻ and wild-type mice (*continued*).

was separated and stored frozen (-80°C) until assayed for testosterone. Blood plasma testosterone levels were assayed by radioimmunoassay (RIA) using an ^{125}I kit purchased from ICN Biochemicals (Carson, CA). The testosterone assay was highly specific; cross-reactions with other steroid hormones were $<0.1\%$. The plasma testosterone values were determined in a single RIA. Because the SEM exceeded the mean, a log-transform of the data was performed and statistical analysis of these transformed data was conducted. Many tests were used to assess coordinated behaviors, including orienting reactions, forelimb strength, coordination, climbing, and locomotion. All sensorimotor tests were scored blindly. In one test of agility, the time required to turn in a blind alley (up to 2 min) was recorded. The mouse was placed facing the back wall of an alley (3 cm wide with walls 15 cm high). There was no difference between nNOS⁻ and wild-type mice in this task (22.0 ± 0.2 versus 22.3 ± 0.3 s, respectively; $P > 0.05$). To assess locomotor balance and coordination, a mouse was placed at the center of a wooden "bridge" 60 cm long suspended 60 cm above a foam pillow. A pole bridge (2 cm in diameter) was used and the time taken for each mouse to reach the platform on either end of the bridge within 120 s was recorded. If the mouse fell, then the latency to fall within 120 s was recorded. There were no significant decreases in the performance of nNOS⁻ mice as compared to wild-type mice. To assess forelimb strength, a mouse was suspended by its forelimbs on a wire stretched between two posts 60 cm above a foam pillow. The time (s) until the mouse fell or 90 s had passed was recorded (a score of zero was assigned to animals that fell immediately; a score of 90 was given to mice that did not fall). There were two trials to this task. nNOS⁻ mice were not significantly impaired as compared to the performance of wild-type mice ($P > 0.05$). Open field activity is a commonly used measure of anxiety levels. To assess open field activity, an animal was placed in an open area (1 m^2) for 10 min. An observer recorded the movement of the mouse during the testing period. A border 4 cm from the wall was marked off and the space beyond the border was operationally defined as the open field. The amount of time spent in the open field was compared to the time the mouse was moving along the wall of the arena. There were no significant differences between nNOS⁻ and wild-type males (9.14 versus 8.99 min, respectively; $P > 0.05$) in their open field behavior.

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Part VIII

D-AMINO ACIDS AS NEUROTRANSMITTERS

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COMMENTARY

Disruptive Science: Incongruent Findings Lead to Novel Insights Into How the Brain Works

Joseph T. Coyle

While scientific research is assumed to be neutral, objective, and dispassionate, the record demonstrates a clear herd mentality among scientists, often leading to the elaboration of unassailable theories. Neuroscience is no different. Dale's law (neurons use only one neurotransmitter), the absence of neurogenesis in the mature brain, and the passive role of glia are some of the truisms that have resisted assault but ultimately have fallen. As the truisms crumble, new vistas are opened to reveal opportunities previously unappreciated, such as brain repair in adulthood or glia as therapeutic targets.

Perhaps guided by the admonitions of his mentor Julius Axelrod ("don't work in crowded areas" and "be the firstest with the mostest"), and powered by his own genius, Solomon Snyder has been smashing neuroscience shibboleths for his entire career. The three papers selected address his research on D-amino acids as novel signaling molecules in the brain.

One of the first facts learned in introductory biochemistry is that eukaryotic cells utilize only L-amino acids. Any D-amino acids found in mammalian tissue therefore must be a by-product of dietary consumption, since plants and bacteria do synthesize D-amino acids. However, a nagging conundrum for over 40 years has been that D-selective enzymes—D-amino-acid oxidase (D-AAO) and D-aspartate oxidase—are expressed in mammalian tissues, with particularly high levels found in the brain (1). The reason for their existence in the brain was unclear, but some speculated that they protected it

from dietary D-amino acids, which were known to disrupt protein synthesis and metabolism (2).

More than a decade ago, D-serine was shown to be present in the rat brain with an uneven regional distribution (3). Interestingly, D-serine was also found to serve as a more potent agonist at the glycine modulatory site on the N-methyl-D-aspartate (NMDA) receptor than glycine itself. This site must be occupied in order for glutamate to open the NMDA receptor channel. Thus, glycine and D-serine have been suggested to be “co-transmitters” with glutamate at the NMDA receptor. Seizing the opportunity to definitively localize D-serine in the brain, Snyder with his colleagues Schell and Molliver generated antibodies against D-serine that was coupled to bovine serum albumin with glutaraldehyde. They demonstrated that these antibodies reacted in a highly specific fashion to a D-serine-glutaraldehyde protein complex (4). After “fixing” rat brain by perfusion with glutaraldehyde, which cross-links amino acids, they showed a remarkably distinct pattern of immunostaining for D-serine in brain, with high levels in cortex, hippocampus, and striatum and virtually undetectable levels in the brain stem and cerebellum. This distribution was the mirror image of D-AAO, raising the possibility that D-AAO determines D-serine levels. Furthermore, they showed that the punctate localization of D-serine reflected its concentration in astrocytic processes—and not in neurons, where most would have assumed that this co-neurotransmitter should be found.

Snyder along with Schell and Cooper used the same immunocytochemical strategy successful in localizing D-serine to identify the cellular localization of D-aspartate, which has also been known to be present in the brain (5; Chapter 21 in this volume). They found that unlike D-serine, D-aspartate had a predominantly neuronal localization in brain in highly selective areas such as the septum, olfactory bulb, and hypothalamus. There was a reciprocal relationship with its catabolic enzyme, D-aspartate oxidase, which is also expressed in neurons. D-Aspartate is also localized in glandular cells such as the pineal and adrenal medulla. While the precise function of D-aspartate remains poorly understood, it does activate NMDA receptors and appears to be synthesized in mammalian cells. Intriguing interactions of D-aspartate with hypothalamic neuroendocrine function suggest that Snyder may be on to something that is not simply *outré* but important (6).

That question about the source of D-serine in brain was resolved with the successful cloning from the mouse brain of a cDNA encoding the enzyme that synthesizes D-serine, serine racemase (SR). This was the first demonstration of a D-amino-acid synthetic enzyme in eukaryotes (7; Chapter 22). The mouse SR interestingly exhibited modest homology to D-amino acid-synthesizing enzymes cloned from prokaryotes. Transfecting cells that do not synthesize D-serine with the SR cDNA conferred on these cells the ability to

synthesize D-serine. Expression of SR was shown to be restricted to brain and liver, although SR protein levels are much higher in brain. Finally, regional SR expression in rat brain corresponded relatively well with the distribution of D-serine immunoreactivity, further supporting the inference that SR is the source of brain D-serine. Notably, SR is highly localized to astrocytes.

Other work from the Snyder laboratory suggested that SR was under the dynamic control of glutamate acting at non-NMDA receptors on astrocytes. In a subsequent study, they worked out the molecular mechanisms and demonstrated their physiological relevance (8; Chapter 23). Using the yeast two-hybrid strategy, which identifies proteins that bind to each other, they demonstrated that the carboxy-terminal of SR has a consensus amino acid sequence that binds to a so-called PDZ 6 domain found in a protein called GRIP (glutamate receptor interacting protein 1). This amino acid sequence in SR permits its binding to and activation of D-serine synthesis by GRIP. GRIP was previously identified as a protein that binds to the AMPA receptor, which they showed is colocalized with GRIP and SR in astrocytes. Furthermore, they demonstrated that these interactions are physiologically important because AMPA receptor activation increases D-serine synthesis in a glial line in a GRIP-dependent fashion.

Directional activation of NMDA receptors by a glutamate gradient has been shown to stimulate granule cell migration along the Bergmann glial cells in the developing cerebellum from the external granule layer to the internal granule layer (9). Since the Bergmann glial cells express high levels of SR, Snyder and colleagues wondered whether D-serine was required for this migration. The hypothesis was confirmed by showing that perfusion of the acute cerebellar slice with soluble D-AAO, which degrades extracellular D-serine, arrested granule cell migration. Furthermore, consistent with the mechanism of SR activation that they discovered, GRIP and AMPA receptor activation also stimulated the migration of the granule cells. Notably, since these migrating granule cells have no synaptic contacts, a novel chemoattractant process is involved that requires a cooperative interaction between the glutamate-stimulated release of D-serine from Bergman glial cells and the activation of granule cell NMDA receptors by glutamate released from neurons, presumably located at their destination in the internal granular layer.

NMDA receptors are central to the plastic changes in brain neurocommunication that are responsible for such fundamental processes as learning, memory, and addiction. Phase-specific activation of NMDA receptors accounts for the persistent use-dependent changes in the efficacy of excitatory neurotransmission known as long-term potentiation and long-term depression. Furthermore, persistent activation of postsynaptic NMDA receptors causes the elaboration of new synaptic spines, thereby increasing the influence of particular excitatory circuits. To restrict the powerful effects of NMDA

receptor activation, the receptor channel is voltage dependent, becoming functional only with substantial membrane depolarization. Typically, this obtains when multiple, convergent glutamatergic inputs fire in close temporal proximity. Snyder's findings place another, novel constraint on NMDA receptor activation at the synapse, the availability of D-serine released from the end-feet of astrocytes surrounding the synapse. This is an active process, as they have shown, driven by synaptic glutamate stimulating AMPA receptors on the astrocyte to activate SR to synthesize D-serine.

Ironically, recent findings with D-serine may have created fissures in a widely held hypothesis proposed by Snyder 30 years ago: the dopamine hypothesis of schizophrenia (10). Genetic findings have linked the gene encoding D-AAO and a protein that modulates its activity to the risk of schizophrenia. Furthermore, D-serine levels have been reported to be reduced in serum and cerebrospinal fluid from patients with schizophrenia. Clinical trials with glycine and D-serine have demonstrated significant improvement in the negative symptoms and in cognition in schizophrenic patients receiving concurrent antipsychotic drugs, the very symptoms provoked by NMDA receptor antagonists in normal subjects. Other strong candidate genes for schizophrenia such as neuregulin, dysbindin, and proline oxidase are only two degrees of separation from the NMDA receptor. But Snyder's pathbreaking research on D-serine provides a whole new context for understanding the pathophysiology of schizophrenia that focuses on glia, not neurons, and on a D-amino acid, not a biogenic amine. *Sic transit gloria*. Disruptive science, albeit unsettling, gets us closer to what is really happening in the brain.

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CHAPTER 21

D-Aspartate Localizations Imply Neuronal and Neuroendocrine Roles

Michael J. Schell
Odelia B. Cooper
Solomon H. Snyder

Although D-amino acids are well known in bacterial physiology, only recently have they been found in mammals (1, 2, 3). Substantial levels of D-serine occur in brain tissue with a regional distribution resembling the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (4). By immunohistochemistry we have shown that D-serine is selectively localized to a subpopulation of astrocytes in close proximity to NMDA receptors and released by glutamate stimulation (5, 6). Since D-serine potently activates the glycine site on NMDA receptors, these findings indicate that D-serine is a novel messenger molecule that serves as an endogenous ligand for this site.

Lajtha and coworkers (1) discovered very high levels of D-aspartate in the brain and other tissues of mammals. D-Aspartate levels are highest in neonatal tissues, attaining millimolar concentrations in newborn rat cerebral cortex, pituitary gland, and 3-week-old adrenal gland (7, 8). Using immunohistochemistry, we now describe selective neuronal localizations of

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D-aspartate in discrete brain areas and endocrine structures. D-Aspartate oxidase (DAPOX), visualized by a novel histochemical technique, is localized inversely to endogenous D-aspartate.

Materials and Methods

Materials

Sprague-Dawley rats were purchased from Sasco (Wilmington, MA). Hypophysectomized rats were from Charles River Breeding Laboratories. Mice (129/SvEv Agouti strain) were from Taconic Farms. Antibody to L-aspartate (9) was from Chemicon. Glutaraldehyde (GA) was from Electron Microscopy Sciences (Fort Washington, PA). The peroxidase Elite staining kit was from Vector Laboratories. All other reagents were from Sigma.

Antibody Production and Purification

D-Aspartate was coupled to BSA with GA and then reduced with NaBH_4 (10). Rabbits were immunized using a colloidal gold technique (5, 11), and stereoselective, high-affinity antiserum was produced after 7 weeks. Before use, batches of D-aspartate antiserum were diluted 1:10 with 0.05% NaN_3 and 10 mM Tris (pH 7.4). Diluted serum (10 mL) was mixed with 1 mL packed agarose beads that had been coupled to GA-BSA at a concentration of 4.3 mg/mL with respect to BSA. Antiserum and beads were incubated for 2 hr at room temperature on a rotating wheel. This incubation was followed by another against L-glutamate-GA-beads for 2 hr. These steps removed antibodies against carrier protein and fixative. Polyclonal antiserum against L-aspartate- thyroglobulin-GA conjugates was negatively purified against GA-treated thyroglobulin beads and used at a 1:500 dilution in the presence of 0.2 mM D-aspartate-GA conjugate.

Immunohistochemistry

Sprague-Dawley rats or Agouti mice were anesthetized with an overdose of sodium pentobarbital and perfused through the aorta for 30 sec with 37°C oxygenated Krebs-Henseleit buffer and then switched to 37°C 5% glutaraldehyde/0.5% paraformaldehyde containing 0.2% $\text{Na}_2\text{S}_2\text{O}_5$ in 0.1 M sodium phosphate (pH 7.4). The perfusion flow rates and volumes were adjusted for the size of the animal, ranging from 20 mL/min and 150 mL for mice (20-gauge needle) to 60 mL/min and 450 mL for adult rats (cannula). After a 20-min delay, brains were removed, trimmed, and postfixed in the same buffer for 2 hr at room temperature. After cryoprotection for 2 days at 4°C in 20% glycerol, 1% NaCl, 0.01% thimerosal, and 50 mM sodium phosphate (pH 7.4), brain sections (40 μm) were cut on a sliding microtome. Floating brain sections were stained using an avidin-biotin peroxidase technique, as

described (5). Because of the high concentrations of L-aspartate in brain, we carried out all D-aspartate antibody incubations (1:2,000 dilution) in the presence of 0.2 mM L-aspartate-GA liquid-phase conjugate (5, 12).

DAPOX and Norepinephrine Histochemistry

Sodium pentobarbital-anesthetized rats were perfused with 37°C Krebs-Henseleit buffer and then 4°C 2% paraformaldehyde in 0.1 sodium phosphate or 4°C 2% glutaraldehyde/0.5% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). After a 20-min delay, tissue was removed, trimmed, and post-fixed on ice for 2 hr. Sections (40 μ m) were cryoprotected overnight, frozen, and then cut on a sliding microtome. To visualize DAPOX, we modified a technique for D-amino acid oxidase (13). Floating brain sections were incubated in the dark at 37°C in 0.1% horseradish peroxidase, 0.01% diaminobenzidine, 0.02% NiCl₂, 0.065% NaN₃, 0.02 mM FAD, and 100 mM Tris preset buffer (pH 8.3), with 20 mM NMDA as a substrate. Specific staining developed after 2–18 hr of incubation, and no staining was observed if L-aspartate was added instead. GA-fixed norepinephrine was visualized as described (14).

Analysis of D-Amino Acids

Free amino acids were extracted from tissues, derivatized with fluorescent chiral reagents, and quantified by HPLC (15).

Results

We developed antiserum to D-aspartate as it would occur in fixed tissues by immunizing rabbits with a GA conjugate of BSA and D-aspartate coated onto colloidal gold particles. We examined the specificity and sensitivity by coupling various amino acids and peptides to ovalbumin with GA and spotting preparations onto nitrocellulose. Dot blots were probed with a 1:5,000 dilution of the antiserum (Figure 21–1). The antibody detected 0.1 nmol of D-aspartate conjugate, while no reactivity was apparent with L-aspartate, L-glutamate, or a variety of aspartate-containing peptides. Slight cross-reactivity (1%) was observed with L-serine-O-sulfate. Preabsorption of the antibody with 0.2 mM D-aspartate-GA conjugate abolished staining. In tissue sections the conjugate also eliminated immunoreactivity (not shown).

We first examined the adrenal glands of 3-week-old rats, which are known to contain high levels of D-aspartate (7). The medulla stained intensely, but the cortex was unstained (Figure 21–2, *top*). Staining in the medulla was associated with some but not all chromaffin cells. To discriminate chromaffin cell subtypes, we stained sections with a histochemical method for norepinephrine (Figure 21–2, *bottom*). Norepinephrine occurred in \approx 20% of chromaffin cells and appeared as round clusters (14). D-Aspartate was selectively excluded from norepinephrine cells and instead concen-

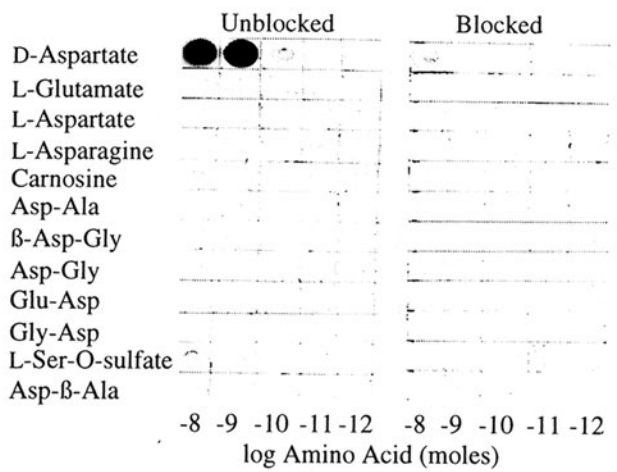


FIGURE 21-1. Antibody specificity test. Ovalbumin was coupled to various amino acids and peptides with GA, reduced with NaBH₄, and then spotted onto nitrocellulose in serial 1:10 dilutions. *Left:* Nitrocellulose was probed with a 1:5,000 dilution of purified antiserum in the presence of 0.2 mM L-aspartate-GA conjugate. *Right:* Blocked incubations included an additional 0.2 mM D-aspartate-GA conjugate. Immunoreactivity was visualized with an alkaline phosphatase secondary antibody. All concentrations are with respect to the amino acid or peptide.

trated in the remaining 80% of the cells, which make epinephrine (Figure 21-2, *top*). By contrast, DAPOX staining was selectively concentrated in norepinephrine chromaffin cells, suggesting that DAPOX destroys endogenous D-aspartate in these cells.

Using an HPLC technique, we measured D-aspartate levels in the pineal. D-Aspartate levels in many pineal glands were higher than any rat tissue previously reported, with an average value of 1.2 ± 1.6 mM ($n=12$), while levels of L-aspartate were 3.65 ± 0.8 mM. Because the pineal displayed diurnal rhythms in numerous substances, we trained male littermates on a 12-hr light/dark cycle for 3 weeks and then sacrificed an animal every 3 hr throughout a diurnal cycle. We found 20-fold variations in D-aspartate (◆), but there was no diurnal rhythm (Figure 21-3, *left*). Conceivably the variations reflect changes on a shorter ultradian cycle or simply random fluctuations. By contrast, L-aspartate levels (□) displayed diurnal rhythmicity, with levels doubling during the dark cycle, and decreasing during light. The pineal gland stained intensely for D-aspartate. In some rats staining occurred in all pineal cells; in other animals staining concentrated in islands of cells near blood vessels (Figure 21-3, *right*). We found no DAPOX staining in the pineal.

In the pituitary gland, D-aspartate was concentrated in the posterior lobe,

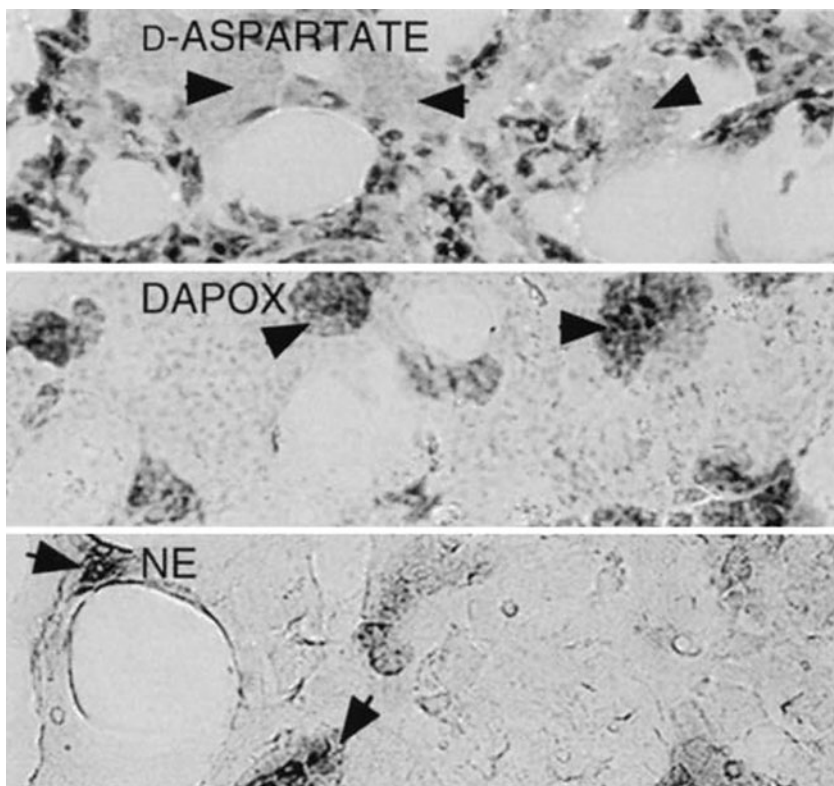


FIGURE 21-2. Localization of D-aspartate (top), DAPOX (middle), or norepinephrine (NE) (bottom) in the adrenal gland.

For the D-aspartate localization, 3-week-old animals were used. Arrowheads point to NE-producing chromaffin cells, which appear in round clusters and are completely unstained for D-aspartate, but densely stained for DAPOX and NE.

with a few stained cells widely scattered in the intermediate and anterior lobes (Figure 21-4A). In the posterior lobe, extremely intense staining was observed in nerve processes and terminals, which derive primarily from cells in the supraoptic and paraventricular nuclei of the hypothalamus. Indeed, intense and highly localized staining for D-aspartate occurred in most magnocellular neurons of both nuclei (Figure 21-4C and D). By contrast, these nuclei stained only faintly with an antibody to L-aspartate and had little DAPOX activity (not shown). The median eminence, which contains axons of magnocellular neurons, was also enriched in D-aspartate and devoid of DAPOX. In the pituitary, DAPOX activity occurred exclusively in the intermediate lobe, with staining concentrated in the outermost cells, adjacent to the anterior lobe (Figure 21-4B).

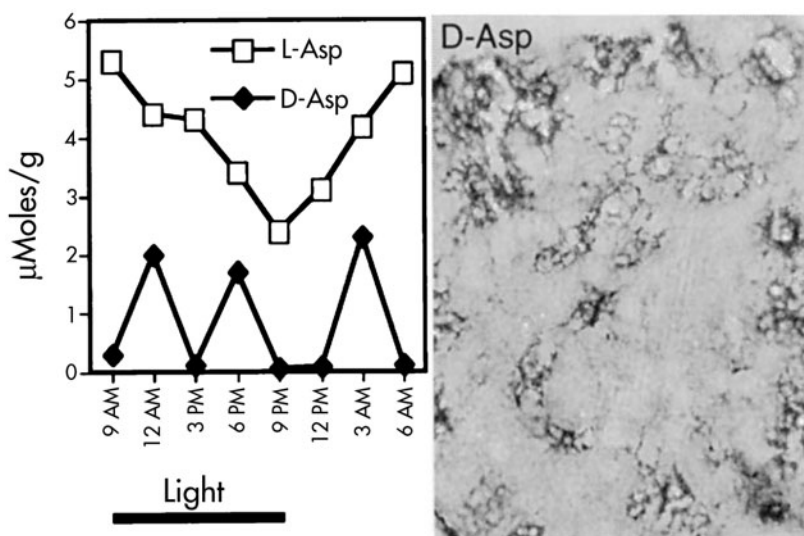


FIGURE 21-3. Disposition of D- and L-aspartate in the pineal gland.

Left: Male rat littermates were trained on a 12-hr light/dark cycle for 3 weeks. One pineal gland was harvested every 3 hr of the cycle and analyzed by HPLC for amino acids. *Right:* Immunohistochemical localization of D-aspartate in the pineal. Some glands exhibited the scattered pattern depicted; in other glands, every cell was intensely labeled.

In the brain (Figure 21-5, *top*), D-aspartate staining was most intense in the external plexiform layer of the olfactory bulb, accessory olfactory nucleus, superior colliculus, medial habenula, and in scattered brain stem nuclei. High densities were also evident in the hypothalamus around the third ventricle, while no staining occurred in the adjacent thalamus. D-Aspartate occurred in the molecular layer but not the granular or white matter layers of the cerebellum. The localization of DAPOX was almost exactly inverse to that of D-aspartate (Figure 21-5, *bottom*). For instance, the hippocampus displayed the weakest staining for D-aspartate and the most intense staining for DAPOX in the brain.

Under higher power examination, highly selective enrichment of D-aspartate but not L-aspartate was evident in large neurons located in lateral septal, triangular septal, and septofimbrial nuclei of 23-day-old male rats (Figure 21-6). Staining was also evident in neurons within the fimbria (Figure 21-7C). These nuclei receive their major inputs from the hippocampus and are thought to produce γ -aminobutyric acid (16). The triangular septal and septofimbrial nuclei provide 90% of all projections to the medial habenula via the stria medullaris, while the lateral septal cells are interneurons. By con-

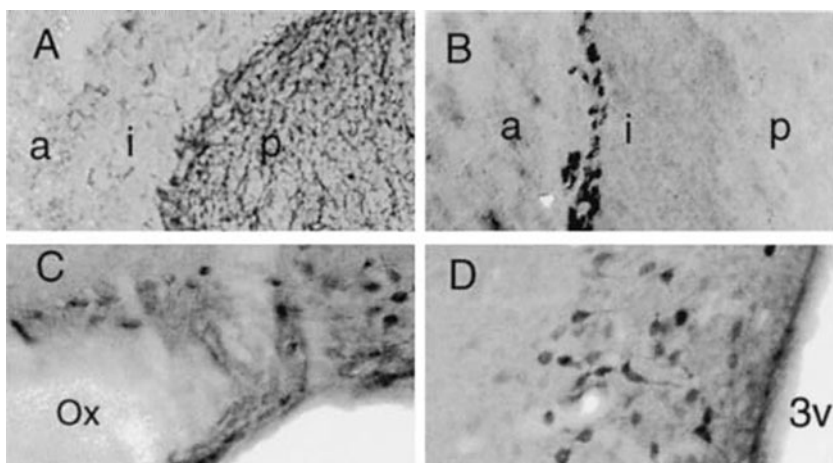


FIGURE 21-4. D-Aspartate and DAPOX visualized in the pituitary and hypothalamic nuclei.

(A) Endogenous D-aspartate in the pituitary is intensely concentrated in the posterior lobe (p), while the intermediate (i) and anterior (a) lobe exhibit very low staining in a few widely scattered cells. (B) DAPOX in the pituitary is restricted to the intermediate lobe (i), with activity concentrated in a narrow band of cells immediately adjacent to the anterior lobe (a). (C) D-Aspartate is concentrated in magnocellular neurons of the supraoptic nucleus of the hypothalamus, near the optic nerve (Ox). (D) D-Aspartate is concentrated in magnocellular neurons of the paraventricular nucleus, near the third ventricle (3v).

trast, the medial septal nuclei and the bed nucleus of the stria terminalis, groups that project to the hippocampus, were unstained for D-aspartate.

Relative to stained cells in the olfactory bulb, hypothalamus, and septum, neurons in the brain stem were less densely stained; however, certain nuclei exhibited staining above background. These consisted mainly of magnocellular groups, the interpeduncular nucleus, inferior olive, cochlear nuclei, gigantocellular cells of the reticular nucleus, and cranial nuclei, especially the hypoglossal and facial. Many of these groups also stained intensely for L-aspartate and/or are known to contain aspartate aminotransferase (17).

Substantial DAPOX activity occurs in most brain areas (18). The most intense staining occurred in the choroid plexus and ependyma (Figure 21-6C), which did not stain for D-aspartate but stains intensely for L-aspartate (19). At high magnification, DAPOX was concentrated in most pyramidal neurons in the cerebral cortex and hippocampus (Figure 21-6A) as well as granule cells of the dentate gyrus. Olfactory receptor neuron cell bodies in the olfactory epithelium neurons also displayed intense DAPOX activity

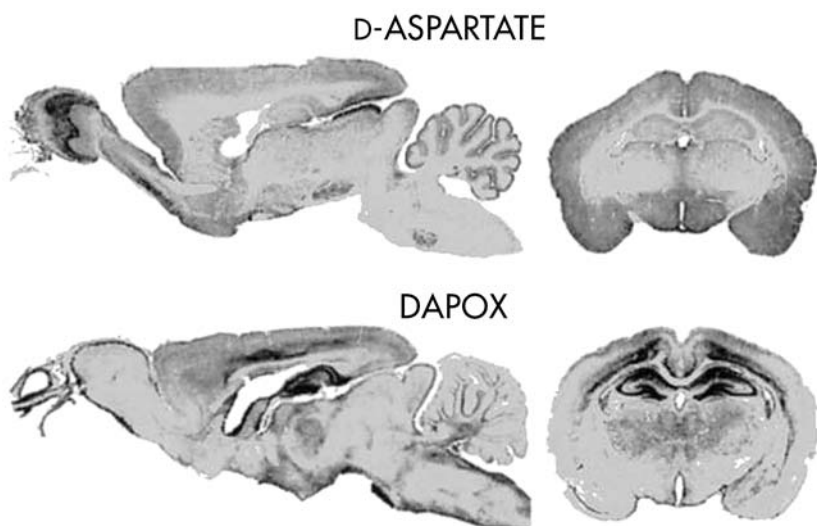


FIGURE 21-5. Inverse brain localizations of D-aspartate and DAPOX.

D-Aspartate is concentrated in the olfactory bulb mitral cell layer, hypothalamus, anterior olfactory bulb, superior colliculus, the molecular layer of the cerebellum, and scattered nuclei in the brain stem. By contrast, DAPOX is concentrated in olfactory receptor neuron layer and glomeruli, hippocampus, cerebral cortex, thalamus, and cerebellar granule cells.

(Figure 21-6B). Within the olfactory bulb, extremely intense DAPOX activity was observed in the glomeruli (Figure 21-8, *right*), which contained terminals of the olfactory neurons, suggesting that DAPOX occurred throughout these cells. By contrast, high densities of D-aspartate occurred in the adjacent external plexiform layer (Figure 21-8, *left*).

A reciprocal relationship between D-aspartate and DAPOX was also evident in the cerebellum (Figure 21-9). D-Aspartate was localized to a subpopulation of basket and stellate cells (Figure 21-9, *left*), but was not contained in Purkinje cells or glia. This staining was most prominent in 3-week-old rats, and could be further enhanced by removal of the pituitary. Four weeks following removal of the pituitary gland from 4-week-old animals, basket and stellate cells were intensely stained for D-aspartate, especially cells with processes in close vicinity to the pia. By contrast, sham-operated animals exhibited no staining (not shown). No D-aspartate was evident in the granule cell layer, whereas DAPOX staining was observed in the granule cell layer and white matter, but not the molecular layer (Figure 21-9, *right*).

Biochemical studies have detected substantial D-aspartate in adult mouse cerebral cortex (3), but not rat (8). We confirmed this species difference bio-

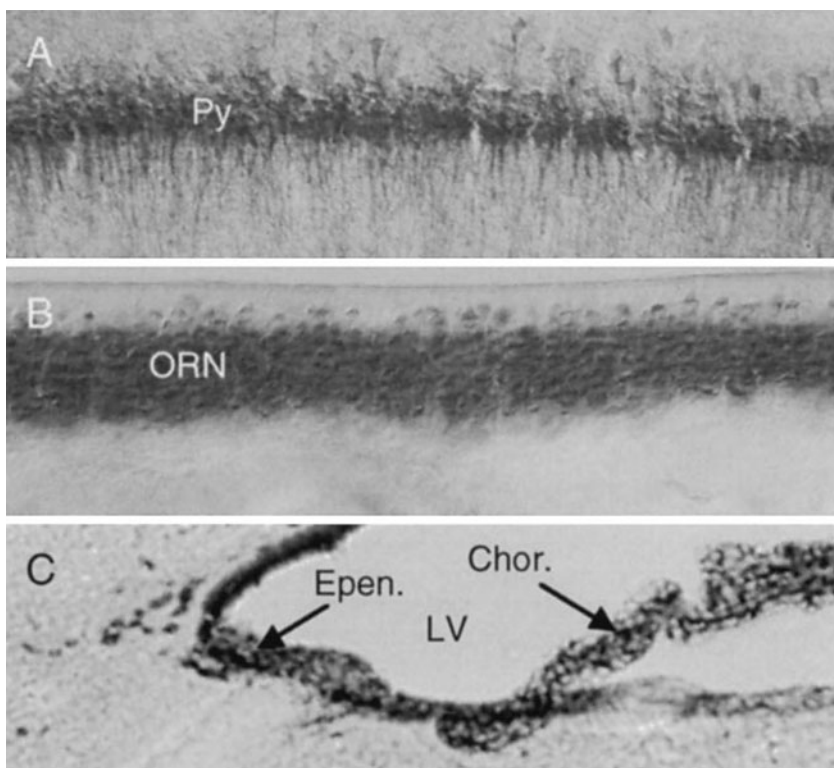


FIGURE 21-6. Cellular localization of DAPOX.

Intense DAPOX activity occurs in all hippocampal pyramidal cells (Py) (A), olfactory receptor neurons (ORN) (B), and ependymal cells (Epen.) and choroid plexus (Chor.) (C). LV, lateral ventricle.

chemically and immunohistochemically. Whereas rat levels in cortex were below 50 nmol/g, mouse levels were about 300 nmol/g. Mouse cerebral cortex and hippocampus stained much more intensely for D-aspartate than rat. Staining in the cortex occurred mainly in pyramidal neurons, especially in superficial layers of piriform cortex. The most dramatic species difference occurred in the hippocampus, where mice exhibited intense staining for D-aspartate in neurons of the CA3 region and hilus (not shown).

Discussion

The principal finding of this study was the selective localization of D-aspartate in discrete neuronal populations in the brain as well as various endocrine organs. L-Aspartate also displays discrete localizations to neuronal populations (9, 19, 20). Low levels of D-aspartate staining occurred throughout the brain

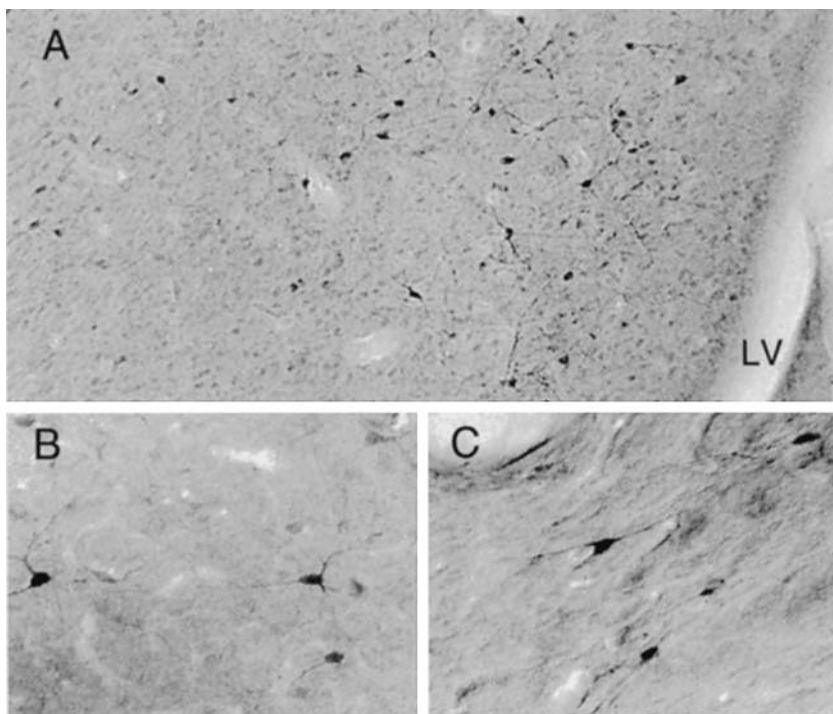


FIGURE 21-7. Localization of D-aspartate in P23 septal neurons.

(A) D-Aspartate occurs in lateral septal nuclei near the lateral ventricle (LV). (B) Higher-power view of multipolar stained neurons in the septum. (C) Localization of D-aspartate in septofimbrial neurons of the fornix.

in a neuronal pattern closely resembling that reported for L-aspartate. Either the antibody had a slight cross-reactivity with L-aspartate in tissue sections, or endogenous D-aspartate existed at low levels in many of the same neurons enriched in the L-isomer. Two regions with intense staining for D-aspartate, the external plexiform layer of the olfactory bulb and the medial habenula, are known to contain high densities of L-aspartate (9, 19, 21).

In most brain regions, D-aspartate localizations appeared to reflect those of L-aspartate minus areas with high densities of DAPOX. This fits with the notion that DAPOX degrades endogenous D-aspartate. Selected brain regions stained much more intensely with the D-aspartate antibody than with the antibody to L-aspartate, and lacked DAPOX activity. These include the supraoptic and paraventricular nuclei of the hypothalamus, septal nuclei, and cerebellar basket/stellate cells of 3-week-old animals. D-Aspartate and DAPOX localizations in mouse and rat brain were similar, but the density of D-aspartate staining was much greater in mouse cerebral cortex and hippocampus than in rat.

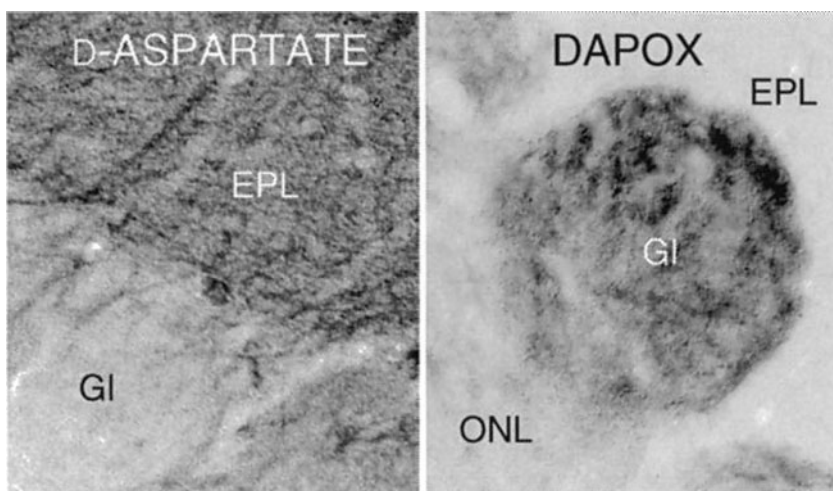


FIGURE 21-8. Inverse localizations of D-aspartate (left) and DAPOX (right) in the olfactory bulb.

EPL, external plexiform layer; GL, glomeruli; ONL, olfactory nerve layer.

D-Aspartate is well known to activate NMDA receptors. Conceivably D-aspartate functions as a neurotransmitter at NMDA or other receptors. If D-aspartate is a neurotransmitter, one would expect depolarization to stimulate its release. Detailed release studies have not yet been conducted. In preliminary experiments, spreading depression elicited by potassium injection into the cerebral cortex of mice caused a pronounced increase in D-aspartate staining in the choroid plexus (M.J.S., M. Eliasson, S.H.S., and M. Moskowitz, unpublished work). This suggests that depolarization elicited by potassium releases D-aspartate, which reaches ventricular fluid and accumulates in choroid plexus to levels great enough to exceed the DAPOX activity.

DAPOX was first described in mammalian tissues more than 45 years ago, but its biological function is unclear (22). DAPOX is a peroxisomal enzyme (23). In the brain, peroxisomes are concentrated in oligodendrocytes in white matter and contain enzymes required for the synthesis of myelin lipids (24). The localizations of DAPOX appear to reflect a unique population of peroxisomes enriched in neurons. In some brain regions, such as the olfactory glomeruli and hippocampus, DAPOX appears concentrated in nerve terminals, suggesting a synaptic function. A synaptic role has been suggested for platelet activating factor, a lipid made exclusively in peroxisomes (25); this lipid could conceivably be made by peroxisomes expressing DAPOX. The reciprocal localizations of DAPOX and D-aspartate are analogous to the recip-

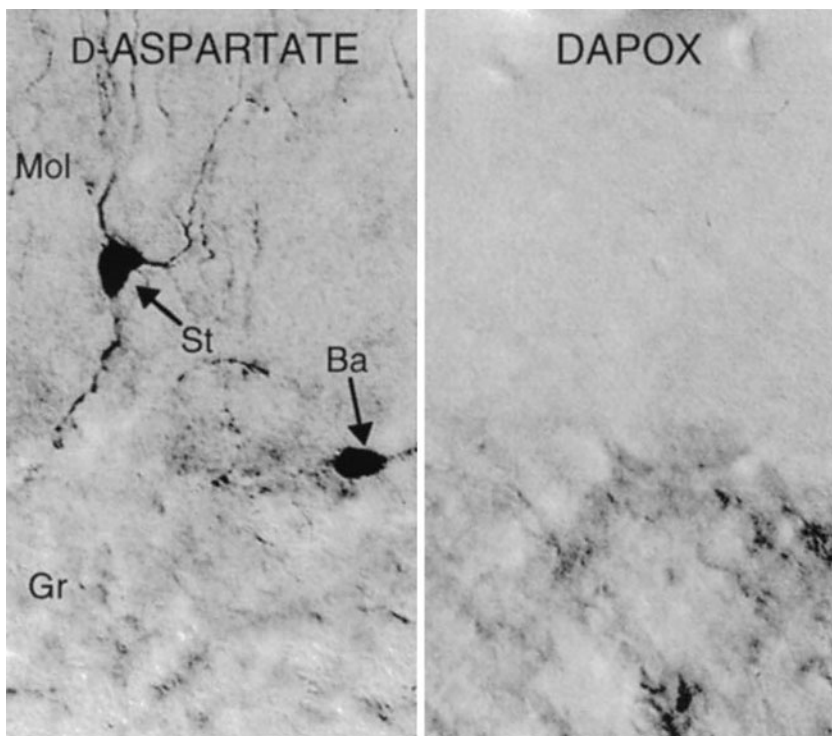


FIGURE 21-9. Inverse localizations of D-aspartate and DAPOX in P23 cerebellum.

Left: D-Aspartate is present in a subset of basket (Ba) and stellate (St) neurons of the cerebellum. *Right:* Cells in the granular layer (Gr) are unstained for D-aspartate, but contain DAPOX.

rocal distributions of D-serine and D-amino acid oxidase, which degrades endogenous D-serine (26). Besides depleting endogenous D-aspartate, DAPOX might inactivate the synaptically released amino acid. For example, hippocampal neurons have intense DAPOX activity and express abundant glutamate transporters, which also carry D-aspartate. Any D-aspartate released synaptically near these cells would be rapidly taken up and degraded.

The anatomical relationships between neuronal groups containing D-aspartate and those containing DAPOX suggest that another role for DAPOX is to degrade D-aspartate in brain regions that might otherwise accumulate D-aspartate via retrograde axonal transport. D-[^3H]Aspartate is a well studied retrograde label for glutamatergic pathways (27, 28, 29). We observed three prominent localizations suggesting that the retrograde transport of D-aspartate occurs endogenously. In the olfactory bulb, D-aspartate is concentrated in the dendrites of mitral and tufted cells, while DAPOX is localized to primary

olfactory neurons and their terminals in glomeruli. D-Aspartate taken up into olfactory receptor neuron terminals would accumulate in these cells if it were not degraded by their intense DAPOX activity. Likewise, hippocampal axons project to the septofimbrial and triangular septal nuclei, which are intensely stained for D-aspartate; any D-aspartate taken up into hippocampal neurons would be degraded by their intense DAPOX activity. A third example occurs in the cerebellum, where basket and stellate cells contain D-aspartate. Parallel fibers, the axons of granule cells located in the molecular layer, could take up released D-aspartate and transport it retrogradely to their cell bodies in the granular layer, which contain DAPOX.

D-Aspartate localizations in various glands suggest a role in neuroendocrine modulation. In the adrenal gland D-aspartate is selectively concentrated in chromaffin cells that make epinephrine, while DAPOX appears in norepinephrine cells. In the hypothalamus, it is unclear whether D-aspartate occurs primarily in oxytocin or vasopressin cells. In preliminary experiments we injected 23-day-old rats *i.v.* with D-aspartate (20 mg/kg) and stained them 15 min or 8.5 hr later. At early time points, we observed a marked enhancement in staining of the pituitary and median eminence but not in the hypothalamus, whereas at 8.5 hr, staining had increased dramatically in the paraventricular nucleus. This suggests that circulating D-aspartate is selectively accumulated by nerve terminals of the posterior pituitary and retrogradely transported to cell bodies in the paraventricular nucleus.

The very high levels of D-aspartate in the pineal gland varied markedly in an unclear pattern. The variations might reflect an ultradian rhythm or pulsatile alterations in pituitary hormone release. However, hypophysectomy did not alter pineal D-aspartate (M.J.S. and S.H.S., unpublished observations). The pineal gland is a circumventricular organ (30). These highly vascularized structures, which are outside the blood-brain barrier, concentrate *i.v.*-administered L-aspartate (31, 32). In rats injected *i.v.* with D-aspartate, intense staining in the pineal was evident in 15 min. The medial habenula, which contains neurons that project to the pineal gland (33, 34), displayed a prominent increase in D-aspartate staining 8 hr after an *i.v.* injection of D-aspartate, while the lateral habenula was unstained (M.J.S. and S.H.S., unpublished observations). Thus, habenular D-aspartate might derive in part from retrograde transport. Habenular D-aspartate could also accumulate via uptake following release from septal nuclei, which contain D-aspartate and comprise the major input to the medial habenula.

Other selected circumventricular organs are greatly enriched in D-aspartate. The median eminence is intensely stained, but the area postrema is not. Intravenous injections of D-aspartate markedly augment staining in the median eminence. The selectivity of the D-aspartate uptake system is suggested by the lack of staining in the area postrema, another circumventricular or-

gan. By contrast, L-aspartate and L-glutamate accumulate in all circumventricular organs following i.v. administration (31, 32, 35).

In summary, we have localized D-aspartate in rat tissues and find it selectively enriched in neurons and glands, where it might function as a novel messenger. The target for D-aspartate is unclear. Although most glutamate receptor subtypes respond to D-aspartate, brain areas enriched in glutamate receptors, such as the hippocampus, have very low levels of D-aspartate; moreover, a number of neuronal groups enriched in D-aspartate, such as septal neurons and cerebellar basket and stellate cells, produce γ -aminobutyric acid. While the transport systems for most amino acids strongly prefer the L-isomer, those for aspartate are stereoblind, suggesting that uptake of D-aspartate into cells plays an important role in its function. Endocrine systems are attractive candidates for modulation by D-aspartate, since the pituitary, adrenal, and pineal glands contain the highest levels of D-aspartate. Consistent with this notion, a recent study demonstrated that D-aspartate can stereospecifically regulate the production of sex steroids (36). Sex steroids serve important functions in brain as well, and steroid biosynthesis is one possible target for neuronal D-aspartate.

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CHAPTER 22

Serine Racemase

A Glial Enzyme Synthesizing D-Serine to Regulate Glutamate-*N*-Methyl-D-Aspartate Neurotransmission

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Seth Blackshaw

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Recently, free D-serine and D-aspartate have been reported in mammals, especially in the nervous system (1, 2). Using highly selective antibodies we localized D-serine to protoplasmic astrocytes in the gray matter areas enriched in *N*-methyl-D-aspartate (NMDA) receptors for the neurotransmitter glutamate (3, 4). We also showed that glutamate, acting through non-NMDA receptors, releases D-serine from astrocyte cultures (3). NMDA receptors require coactivation at a “glycine site” (5) at which D-serine is up to three times more potent than glycine (6, 7, 8), suggesting that D-serine is an endogenous ligand for this site, and is released by glutamate from astrocytic processes that ensheath the synapse. Extracellular levels of endogenous D-serine are comparable to glycine in prefrontal cortex, whereas in the striatum, extracellular D-serine values are more than twice the values for glycine as measured by *in vivo* microdialysis (9). Direct evidence that D-serine normally mediates

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NMDA transmission comes from experiments showing that destruction of endogenous D-serine selectively by application of D-amino acid oxidase greatly reduces NMDA receptor activation in slices and cell culture preparations (J.P. Mothet, A. T. Parent, H.W., R.O. Brady, Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, and S.H.S., unpublished observations). Characterization of the physiologic role of D-serine requires identifying its biosynthetic mechanism. Using partial amino acid sequence from our purified preparation of an enzyme from rat brain converting L-serine to D-serine (10), we now report cloning and expression of serine racemase and its localization to astroglia.

Materials and Methods

Cloning

Full-length serine racemase was cloned by reverse transcription-PCR from mouse brain mRNA using primers based on mouse expressed sequence tags (ESTs) 615391 and 464586 (GenBank accession numbers AA170919 and AA032965, respectively), which corresponded to the 5' and 3' ends of the gene: forward primer, 5'-ATG TGT GCT CAG TAC TGC ATC TCC-3'; reverse primer, 5'-TTA AAC AGA AAC CGT CTG GTA AGG-3'. Several other mouse ESTs also covered parts of the sequence of serine racemase (GenBank accession numbers A1322578, A1173393, AA833469, and AA197364). The ORF and stop codon were confirmed by an independent 3' rapid amplification of cDNA ends reaction using a primer against 5' untranslated region (5'-AAA CAC AGG AGC TGT CAG C-3'). The mouse serine racemase sequence was deposited in GenBank (accession number AF148321).

Cell Culture and Transfection

HEK293 cells were cultured in DMEM/penicillin-streptomycin/10% FBS media. Cells were transfected with serine racemase constructs subcloned in pRK5-KS vector with a cytomegalovirus promoter (provided by A. Lanahan and P. Worley, Johns Hopkins University) by using the calcium chloride method. Serine racemase mutant K56G was constructed by PCR. Equal expression of wild-type and mutant enzyme was confirmed by Western blot analysis of transfected cells.

D-Serine Synthesis

In studies of D-serine synthesis, cells were cultured in media supplemented with increasing concentrations of L-serine. Unsupplemented media contained 0.4 mM L-serine. L-Serine used in the experiments was rendered free of contaminating D-serine as described (10). D-Serine in cells was measured in 25-mm culture dishes 36 hr after transfection. The cells were washed twice with cold PBS, followed by addition of 5% trichloroacetic acid (TCA)

to extract free amino acids. After removing TCA with diethyl-ether, D-serine content was analyzed by both HPLC and a luminescence method as described (10). For measurement of D-serine in culture media, an aliquot was deproteinized by adding TCA (5% final concentration) and processed as described above for D-serine in cells. Blanks were performed by analyzing medium alone. For measurement of D-serine synthesis in cell homogenates, transfected cells were disrupted by sonication in 10 mM Tris/HCl (pH 8.0), 10 μ M pyridoxal 5' phosphate, and 0.2 mM PMSF. Reactions were started by adding 10 mM L-serine to cell protein extract. After 2 hr at 37°C, the reaction was stopped by addition of TCA 5%. Blanks were performed with heat-inactivated cell extract.

Immunocytochemistry

HEK293 cell cultures were fixed 36 hr after transfection with 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and 0.1% sodium metabisulfite for 40 min. After reduction of free aldehyde groups by 30-min incubation in 0.5% sodium borohydride, cells were incubated with a purified anti-D-serine antibody raised against a reduced glutaraldehyde conjugate of D-serine (3). The antibody detects as few as 10 pmol of D-serine and is 100-fold less sensitive to L-serine. To avoid any cross reactivity with L-serine present in the cells, incubations were performed in the presence of 0.5 mM L-serine-glutaraldehyde conjugate. Preabsorption of the antibody with 0.5 mM D-serine-glutaraldehyde conjugate abolished immunoreactivity. Staining was developed with an immunoperoxidase ABC Elite kit (Vector Laboratories) by using 3,3'-diaminobenzidine as substrate.

Primary Cultures

Mixed neuronal-glial cell cultures were prepared as described (27). Cortical embryonic neuronal cultures (E18) virtually free of glia were prepared in a serum-free media supplemented with B27 (28). Primary astrocyte cultures were prepared from cerebral cortex as described (29). Astrocytes type 1 are polygonal flat cells that adhere strongly to the tissue culture flasks. Type 2 astrocytes are nonadherent process-bearing cells that can be detached by shaking the cultures, leaving the adherent cell population enriched in type 1 astroglia. Although both types of astrocytes strongly express astrocytic markers, the *in vivo* counterparts of type 1 and type 2 astroglia are still controversial, as astrocytes type 2 were not found in the cerebral cortex *in vivo* (30). Control experiments were conducted to assure that the differences observed in serine racemase expression were not caused by differences in components of media used in each culture. Accordingly, we cultured neurons with serum containing media after diminishing glia proliferation with 5-fluoro-2'-deoxyuridine or cultured astrocytes in DMEM/10% FBS/penicillin-strepto-

mycin media. Changes in the media did not alter the expression pattern of serine racemase.

Antibody Production

Polyclonal antibody against his-tagged and bacterially expressed serine racemase was raised in rabbits and affinity-purified as described (31).

Immunohistochemistry

Sprague-Dawley rats were used at 13 days of age. For serine racemase staining, sagittal sections of 10 μ m were cut on a cryostat and fixed by immersion in methanol for 5 min at room temperature. With our antibody, serine racemase immunoreactivity was very low or absent when aldehyde fixatives were used. Partial trypsinization of the tissue fixed with 4% paraformaldehyde restored some of the antigenicity, suggesting that aldehyde fixation blocked access of the antibody to the antigenic epitopes. Purified antibody was used at 0.5 to 1 mg/mL. For D-serine staining, rats were perfused with 5% glutaraldehyde and 0.5% paraformaldehyde and processed as described (3). For double-labeling experiments, brain sections fixed in methanol were incubated with affinity-purified antiserine racemase (0.2 mg/mL) and monoclonal anti-glial fibrillary acidic protein (GFAP) antibodies (Sigma, at 1:800 dilution) for 48 hr at 4°C. Secondary antibodies consisted of a Cy3-conjugated anti-rabbit and a FITC-conjugated anti-mouse used at 1 mg/mL (Jackson ImmunoResearch). Images were captured on a Noram (Philadelphia) confocal microscope.

Results

We obtained amino acid sequence for two peptides from purified serine racemase. A search of the GenBank database revealed identity to two ESTs from the mouse genome encoding fragments of a gene of unknown function. Using primers based on EST sequences, we cloned the full-length mouse serine racemase by reverse transcription-PCR. To confirm that we had cloned the complete ORF and stop codon, we also performed 3' rapid amplification of cDNA ends (RACE) using a primer based on the 5' untranslated region of the gene. A single band was observed in the RACE reaction, which contained a full-length sequence of the enzyme (Figure 22-1A).

Serine racemase comprises 339 aa with a predicted molecular mass of 36.3 kDa (Figure 22-1B). Close to the N terminus we observed a region that has a consensus sequence for pyridoxal 5' phosphate. In this region, serine racemase displays homology with enzymes of the serine/threonine dehydratase family, best represented by rat L-serine dehydratase, the closest mammalian sequence, which, like serine racemase, is a pyridoxal phosphate-dependent enzyme (Figure 22-1C). The overall identity of serine racemase

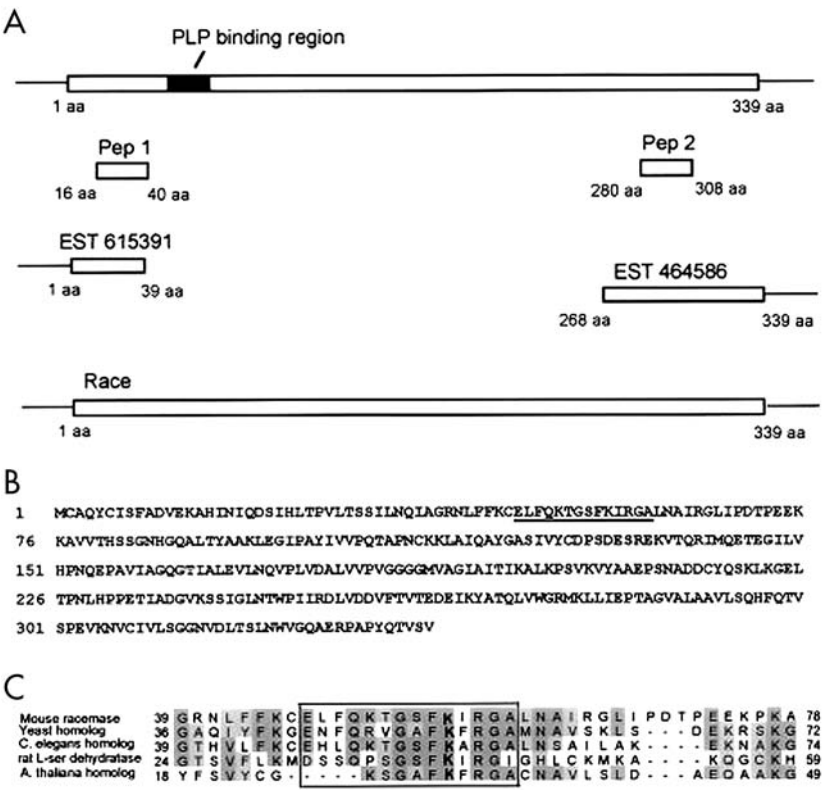


FIGURE 22-1. Cloning and sequence analysis of mouse serine racemase.

(A) Strategy for serine racemase cloning. Two peptides obtained by internal amino acid sequence of purified enzyme (PEP1, HLNIQDSVHLTPVLTSILNQIAGR; PEP2, LLIEPTAGVGLAAVLSQHFQTVSPEVK) corresponded to mouse ESTs in GenBank. Full-length serine racemase cloned by reverse transcription-PCR from mouse brain has a pyridoxal 5' phosphate (PLP) binding region. The ORF and stop codon were confirmed by an independent 3' rapid amplification of cDNA ends (Race) reaction. (B) Amino acid sequence of serine racemase. Underlined sequence corresponds to the amino acid consensus for pyridoxal 5' phosphate binding (Prosite accession number PS00165). (C) Alignment of proteins exhibiting significant homology to serine racemase in the pyridoxal 5' phosphate binding region (boxed area), which include homologs in yeast, *C. elegans*, *A. thaliana*, and rat L-serine dehydratase (GenBank accession numbers P3600, CAB02298, CAB39935, and DWRTT, respectively). The lysine residue predicted to interact with pyridoxal 5' phosphate molecule is in bold, and homologous amino acid residues are shadowed.

to L-serine dehydratase is only 28%. We observed putative homologs of serine racemase with previously unknown function in yeast *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and the plant *Arabidopsis thaliana*, which display 40%, 37%, and 46% overall amino acid sequence identity, respectively to mouse serine racemase (Figure 22-1C). The similarity of the sequence between mammals and plants indicates that serine racemase represents an additional family of pyridoxal phosphate enzymes distinct from serine dehydratase, whose mammalian sequence shows less similarity to mammalian serine racemase than the plant gene.

To establish that the cloned protein displays serine racemase activity, we transfected HEK293 cells that do not possess endogenous D-serine. Augmenting D-serine concentration in the medium led to striking increases in D-serine generation in both cells and culture media (Figure 22-2 A, B). Synthesis of D-serine was specific as HPLC analysis failed to reveal formation of any other D- or L-amino acid. To explore the dependence on pyridoxal 5' phosphate binding, we constructed a mutant in which lysine-56, predicted to bind pyridoxal 5' phosphate in serine racemase (Figure 22-1C), was replaced by glycine (K56G mutant). The mutant enzyme did not catalyze the formation of D-serine when transfected into HEK293 cells (Figure 22-2A, B). We demonstrated enzyme activity in homogenates of the transfected HEK293 cells but not in the K56G mutant (Figure 22-2C). Amino-oxoacetic acid, a known inhibitor of pyridoxal 5' phosphate enzymes, abolished enzyme activity (Figure 22-2C). Cells transfected with serine racemase and fixed with glutaraldehyde stained positively with a stereospecific antibody to D-serine-glutaraldehyde conjugate, whereas mock-transfected cells were unstained (Figure 22-2D, E). Preabsorption of the antibody with D-serine glutaraldehyde conjugate abolished the staining (Figure 22-2F). Intense immunoreactivity was observed in cells that accumulated exogenous D-serine added to media several hours before fixation, demonstrating that the antibody readily recognized D-serine (see Figure 22-2G). Untransfected mixed neuronal-glial primary cultures from rat brain synthesized D-serine with greater synthesis in the presence of higher concentrations of L-serine in culture media, ensuring that the racemizing enzyme activity is responsible for the formation of endogenous D-serine (Figure 22-2H, I).

Northern blot analysis revealed a single message of 2.6 kb with highest levels for serine racemase RNA in the liver, second highest values in brain, low levels in the kidney, and very faint message in the other tissues (Figure 22-3A). Western blot analysis revealed a prominent single band in the brain at about 38 kDa (Figure 22-3B), fitting with the molecular mass of purified serine racemase (10). Serine racemase protein expression was higher in the brain than liver, with very faint or no detectable expression in other tissues (Figure 22-3B). Expression of serine racemase coincides with the high levels

of endogenous D-serine in the brain. Rat liver expresses large amounts of D-amino acid oxidase, which completely metabolizes D-serine in most peripheral tissues where D-serine is almost undetectable (11).

Subcellular fractionation of rat brain revealed the great proportion of serine racemase protein in soluble fractions (Figure 22–3C). To explore the cellular localization, we prepared neuronal cultures virtually free of glial cells, glial cultures greatly enriched, respectively in type 1 or type 2 astrocytes, and neuroblastoma cell line Neuro-2A. Highest levels of enzyme protein were observed in the astrocyte-enriched cultures with lowest levels in the neuronal cultures and the neuroblastoma cell line (Figure 22–3D). The molecular mass of serine racemase in the subcellular fractions and the cultures was essentially the same as in HEK293 cells transfected with serine racemase (Figure 22–3E).

Further evidence that serine racemase accounts for the physiologic biosynthesis of D-serine came from immunohistochemical studies. The distribution of serine racemase was closely similar to that of endogenous D-serine with highest concentrations in the forebrain and negligible levels in the brain stem (Figure 22–4A, B). Whereas the adult cerebellum possesses very low levels of D-serine (3), the 13-day-old rat brain used in our experiments showed substantial expression of serine racemase and D-serine in the cerebellum. At high magnification we observed both serine racemase and D-serine selectively enriched in astrocytes concentrated in gray matter areas. High densities were observed in astrocytes throughout the cerebral cortex (Figure 22–4C, D). In the hippocampal formation, serine racemase and D-serine occurred in astrocytes with highest densities in the hilus of the dentate gyrus (Figure 22–4E, F). The pyramidal cell layers and the granule cell neurons of the dentate gyrus were unstained. In the 13-day-old rat cerebellum, both D-serine and serine racemase were highly enriched in Bergmann glia (Figure 22–4 G, H), where D-serine may play a role in the NMDA transmission that is essential for migration of granule cells in neural development (12). Scattered stellate astrocytes and glial cell processes throughout the granule cell layer also were stained for both serine racemase and D-serine. Staining in cerebellar white matter was much less (data not shown). In the telencephalic white matter, very strong serine racemase and D-serine staining was evident in astrocytes of the corpus callosum (Figure 22–4I, J), which continuously generates new astrocytes in young rats. Direct colocalization by double labeling of serine racemase and D-serine is not feasible because we found the glutaraldehyde fixation used for D-serine staining incompatible with proper preservation of serine racemase antigenicity. However, like D-serine (3), cells staining for serine racemase also stained for GFAP, a marker for astrocytes in double-labeling experiments (Figure 22–5A, B, C, D). We did not detect significant neuronal staining above the background for both

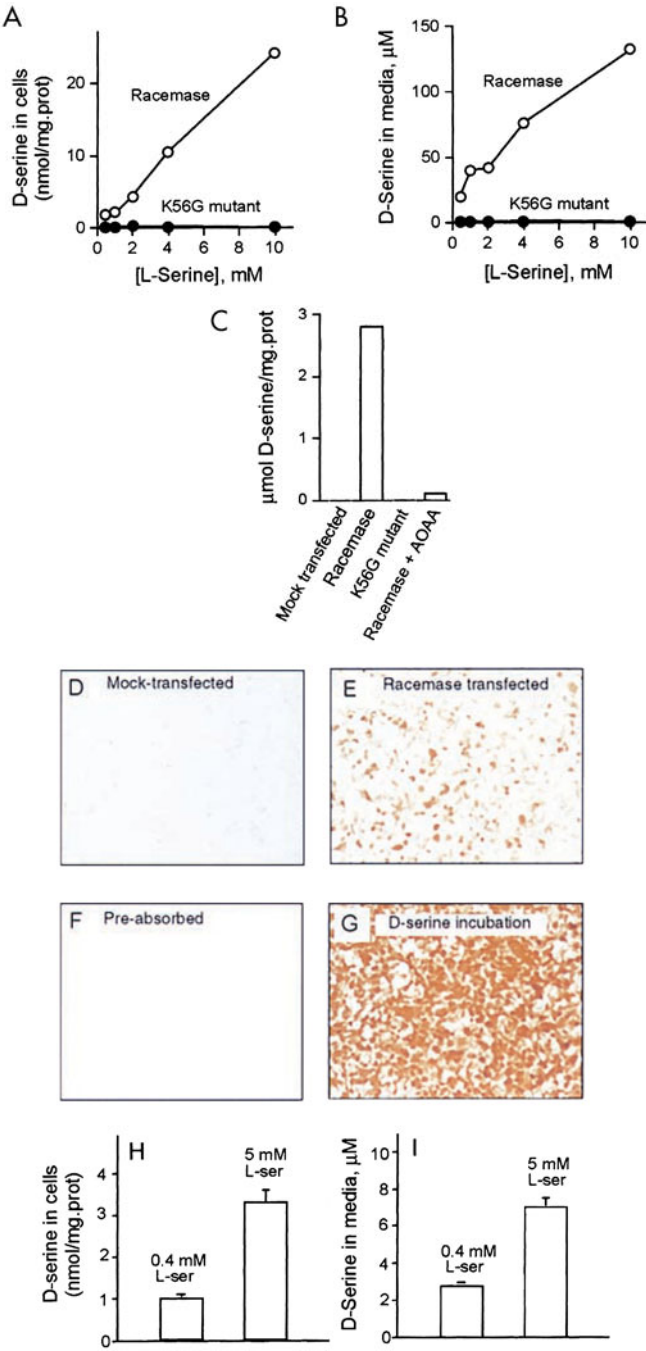


FIGURE 22-2. Serine racemase catalyzes the formation of D-serine *in vivo*.

(A) Analysis of D-serine synthesis in transfected cells. HEK293 cells were transfected either with full-length mouse serine racemase (○) or serine racemase mutant K56G (●). (B) Analysis of D-serine synthesis in culture media from transfected cells. (C) Synthesis of D-serine in cell homogenates. Mock-transfected cells or K56G mutant exhibited no activity, whereas addition of 0.5 mM amino-oxyacetic acid (AOAA) inhibited most of the activity in serine racemase-transfected cell extracts. (D–G) HEK293 cells were transfected with serine racemase and further analyzed for D-serine content by immunocytochemistry. (D) Mock-transfected cells incubated in DMEM media supplemented with 10 mM L-serine. (E) Serine racemase-transfected cells incubated in media supplemented with 10 mM L-serine. (F) Serine racemase-transfected cells incubated in media supplemented with 10 mM L-serine and stained with antibody preabsorbed with 0.5 mM D-serine-glutaraldehyde conjugate. (G) Mock-transfected cells first incubated for 12 hr in media containing 5 mM D-serine. Intense immunostaining represents accumulation of D-serine by the cells, which was not significantly metabolized during the course of the experiment. (H and I) Synthesis of D-serine by mixed neuronal-glia cell culture. A significant increase in D-serine synthesis was observed by supplementing the media with 5 mM L-serine. D-Serine produced in cells (H) and released to culture media (I) was analyzed 48 hr after addition of L-serine. The results are representative of four independent experiments with different culture preparations (A–G) and presented as the mean \pm SEM of three independent experiments (H and I).

serine racemase and D-serine, which is consistent with our observation that neurons express very low levels of serine racemase by Western blot analysis of cultures virtually free of GFAP-positive glial cells (Figure 22-3D). The enrichment of serine racemase in glia indicates that D-serine is formed in the cells that contain endogenous D-serine rather than being synthesized in neurons and transported into glia.

Discussion

Heretofore, demonstrations of D-amino acids in mammalian tissues often had been ascribed to dietary origin or intestinal bacteria (13, 14). Our identification of mammalian serine racemase as the physiologic biosynthetic enzyme for D-serine establishes an endogenous origin. Though several amino acid racemases have been cloned from bacterial sources, no eukaryotic amino acid racemase has been previously cloned. All of the known amino acid racemases have been cloned from archaea or eubacteria, including alanine, aspartate, glutamate, serine, and phenylalanine racemases (15, 16, 17, 18, 19). None of these display significant amino acid sequence homology to serine racemase. Accordingly, the enzyme we have cloned reflects an additional family of

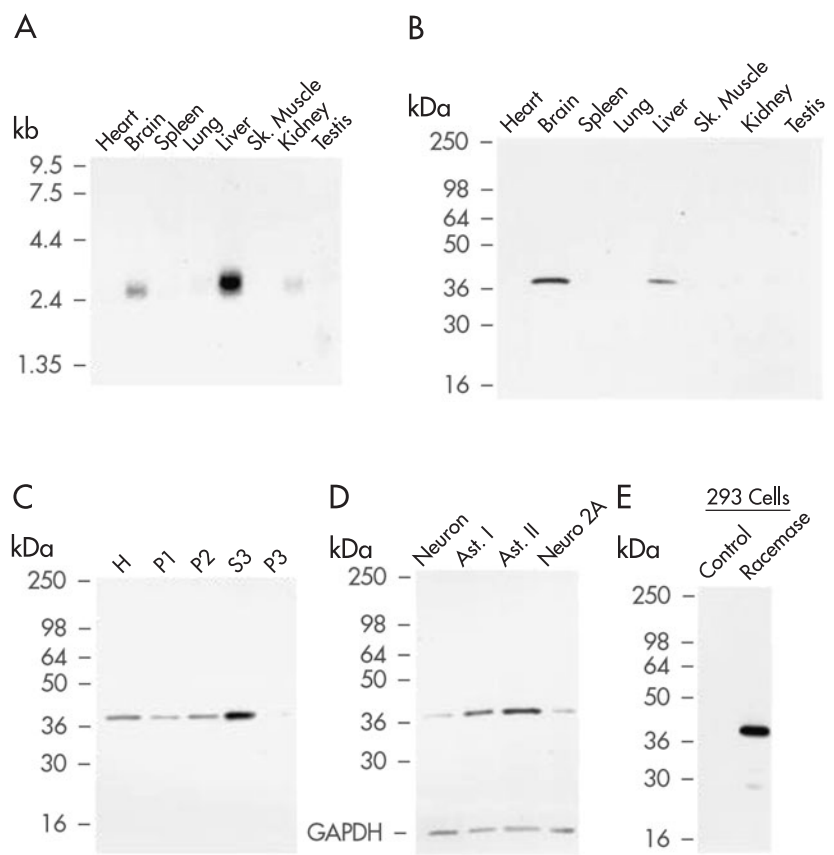


FIGURE 22-3. Distribution of serine racemase.

(A) Northern blot analysis. A full-length serine racemase probe was used to probe a multiple-tissue rat mRNA membrane (CLONTECH). (B) Western blot analysis of serine racemase in rat tissues. Immune serum to serine racemase was used at 1:10,000 dilution. (C) Subcellular fractionation of brain extracts showing enrichment of serine racemase in the cytosolic fraction. H, total homogenate; P1, 9,000g pellet; P2, 30,000g pellet obtained from P1 supernatant; S3, supernatant of 120,000g pellet representing soluble cytosolic proteins; P3, 120,000g pellet. (D) Enrichment of serine racemase in glia. Western blot of serine racemase in neuronal culture virtually free of glia, astrocyte type 1 primary culture (Ast. I), astrocyte type 2-enriched culture (Ast. II), and neuroblastoma (Neuro 2A cells). To assure that the same amount of protein was loaded per lane (20 mg homogenate protein), the blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (E) Western blot of serine racemase in transfected HEK293 cells. Each lane contains 100 ng of homogenate protein.

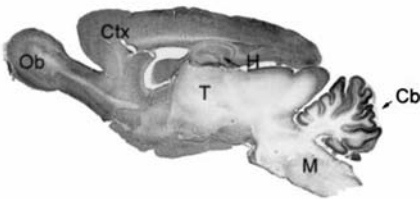
amino acid racemases in eukaryotes. Whether additional mammalian amino acid racemases exist is unclear. The only other D-amino acid consistently observed in significant amounts in mammalian tissues is D-aspartate (20). We localized D-aspartate to selected neuronal populations in the brain and neuroendocrine tissues such as the pineal gland, the posterior pituitary, and the adrenal medulla (21). Biosynthetic mechanisms for D-aspartate have not been elucidated. Failure to detect other D-amino acids in mammalian tissues does not rule out their existence and rapid turnover, which might account for very low or undetectable levels. Our cloning of serine racemase may facilitate the discovery of other mammalian amino acid racemases.

Cloning of serine racemase should greatly enhance characterization of D-serine as a modulator of NMDA neurotransmission. In contrast to glycine, D-serine is selective for NMDA receptors, as it does not activate inhibitory strychnine-sensitive receptors (22). Endogenous levels of D-serine, which are in the micromolar range (9), are adequate to influence the "glycine" site of NMDA receptors for which D-serine is at least as potent as glycine (6, 7, 8). Extracellular D-serine concentration is similar to glycine in the forebrain and up to 2.6 times higher than glycine in the striatum (9). D-Serine is highly localized to astrocytic foot processes that are in close contact with neurons in the synaptic cleft (3, 4), whereas the bulk of endogenous glycine generally is distributed in amino acid pools involved in protein synthesis and inhibitory synapses.

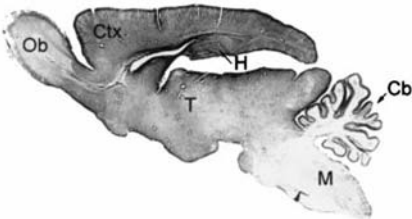
Recently, we showed that purified D-amino acid oxidase, which is virtually absolutely selective for D-serine as opposed to L-amino acids, depletes endogenous D-serine but no other amino acids from brain slices and cultures. Destruction of endogenous D-serine selectively by application of D-amino acid oxidase greatly reduces NMDA receptor activation (J.P. Mothet, A.T. Parent, H.W., R.O. Brady, Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, and S.H.S., unpublished observations). Moreover, application of D-serine was found to be 100 times more potent than glycine in enhancing the NMDA component of miniature end-plate synaptic currents recorded in rat hypoglossal motoneurons (23). These findings, taken together with the closely similar regional localizations of D-serine and NMDA receptors (4), strongly imply that D-serine is a major endogenous ligand for the glycine site of NMDA receptors.

The cloned-expressed serine racemase should help clarify biological roles of D-serine. Selective inhibitors of serine racemase should be valuable tools for investigating the regulation of NMDA transmission. Massive release of glutamate to stimulate NMDA receptors after stroke is thought to be responsible for the major portion of neural damage (24). Drugs that block the glycine site of NMDA receptors prevent stroke damage (25, 26). Inhibitors of serine racemase may provide novel therapeutic agents for stroke and other neurodegenerative diseases in which glutamate excitotoxicity plays a pathophysiologic role.

A Serine Racemase



B D-serine



Serine racemase

D-serine

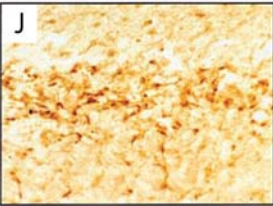
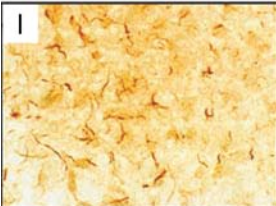
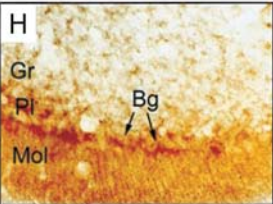
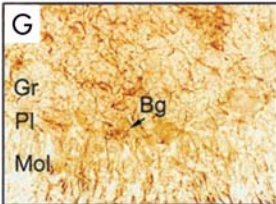
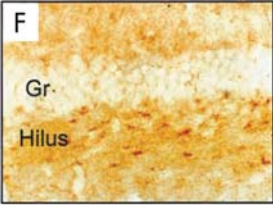
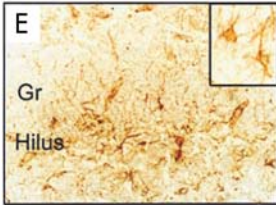
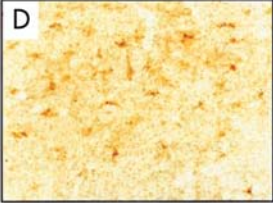
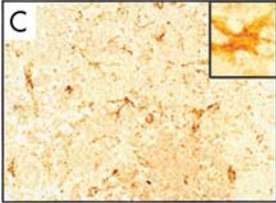
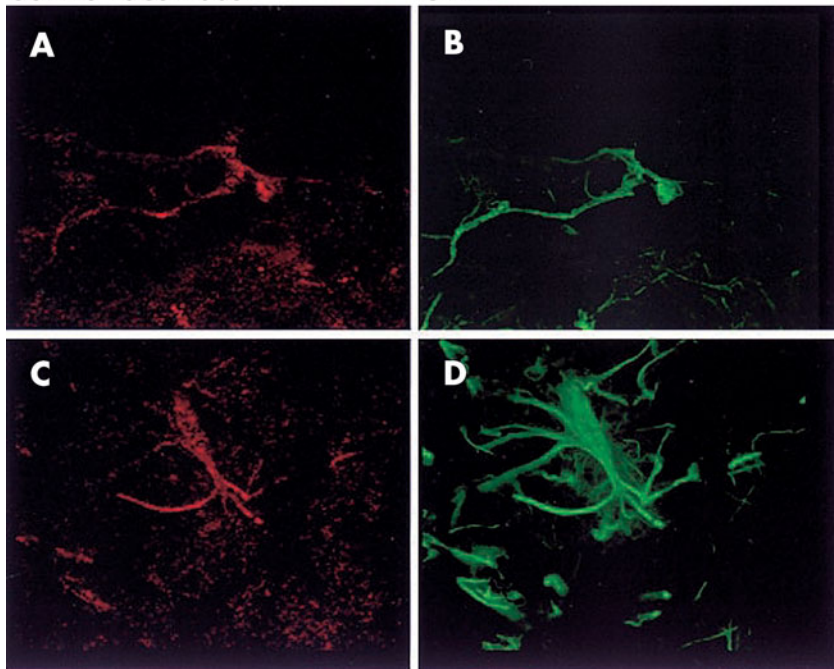


FIGURE 22-4. Colocalization of serine racemase and endogenous D-serine in brain.

(A) Immunohistochemical staining for serine racemase. (B) Immunohistochemical staining for D-serine. In the cerebral cortex (C and D), both serine racemase and D-serine are evident in glial cells with morphology corresponding to astrocytes. (C, *inset*) A $\times 1,000$ magnification of a glial cell positive for serine racemase in the cerebral cortex. In the hippocampus (E and F), several glial cells containing serine racemase and D-serine occur in the hilus of the dentate gyrus. Granule cell neurons (Gr) are unstained. (E, *inset*) A $\times 650$ magnification of glial cells positive for serine racemase in the hippocampus. In the cerebellum (G and H), Bergmann glia cells (Bg) in the Purkinje cell layer (Pl) and scattered stellate astrocytes and glial processes in the granule cell layer (Gr) are positive for both serine racemase and D-serine. Arrows depict Bergmann glia cell bodies that extend processes toward the pia in the molecular layer (Mol) of the cerebellum. In the corpus callosum (I and J), astrocytes are strongly labeled for both serine racemase and D-serine. Though both serine racemase and D-serine occur in the same types of cells, subtle morphologic differences are noticeable, perhaps reflecting the use of different fixatives and tissue processing for each staining. Except for the insets, magnifications are $\times 400$.

Serine racemase**GFAP****FIGURE 22-5. Colocalization of serine racemase and GFAP in brain.**

Serine racemase (red) colocalizes with GFAP (green) in double-labeling experiments. Astrocytes of different regions and different shapes are strongly labeled, including accessory olfactory bulb (A and B) and hindbrain (C and D). Pictures were taken at $\times 1,000$ magnification.

The sequence reported in this paper has been deposited in the GenBank database (accession no. AF148321).

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CHAPTER 23

Serine Racemase

Activation by Glutamate Neurotransmission via Glutamate Receptor Interacting Protein and Mediation of Neuronal Migration

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The NMDA receptor is activated by the neurotransmitter glutamate as well as a second agonist influencing a site that can be stimulated by glycine (1). Recent studies indicate that D-serine is a major, if not principal, ligand for this "glycine site" (2, 3, 4, 5). D-Serine is formed by serine racemase (SR) from L-serine (5). SR and D-serine are localized to a population of protoplasmic astrocytes that ensheath synapses and possess the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor. SR also occurs in radial glia such as Bergmann glia that guide cerebellar granule cells migrating from the external granule layer (EGL) to the internal granule layer (IGL) during cerebellar development (6, 7). This process, one of the best characterized instances of neuronal migration, has been perplexing, because immature granule cells lack synaptic contacts. Moreover, whereas their migration is determined by glutamate acting via NMDA receptors, the cellular interactions that mediate the neuronal migration have not heretofore been identified (7).

We report that SR binds to the AMPA receptor binding protein GRIP (glutamate receptor interacting protein), leading to markedly enhanced SR activity and D-serine release from glia, actions that are elicited by AMPA receptor stimulation. Overexpression of GRIP in mice also augments NMDA receptor-mediated neuronal cell migration. In brain slices we demonstrate that D-serine is required for granule cell migration via chemokinetic influences on granule cells.

Materials and Methods

Yeast Two-Hybrid Screening

Yeast two-hybrid screening used Y190 yeast strains, which contain HIS3 and β -gal reporter genes. Full-length SR gene was subcloned into pPC97, containing GAL4 DNA binding domain. Rat hippocampus and cortex cDNA library was cloned into pPC86, containing GAL4 transactivation domain. More than one million clones were transformed and screened. All other constructs of SR and GRIP were subcloned into pPC97 or pPC86.

Protein Binding Assays

SR(WT) and SR(V339G) tagged with GST were transfected into human embryonic kidney (HEK)-293 cells by using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were harvested in lysis buffer [50 mM Tris-HCl, pH 7.4/50 mM NaCl/1 mM DTT/1 mM EDTA/1 \times PICS (protease inhibitor mixture) tablet (Roche, Basel, Switzerland)/1 mM PMSF]. Cell lysate was incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia) at 4°C for 1 h, and the beads were washed with wash 1 (lysis buffer plus 0.1% Triton X-100) and wash 2 (250 mM NaCl, 1 mM EDTA in 50 mM Hepes, pH 7.4) three times.

Rat brains were homogenized in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4/50 mM NaCl/2 mM EDTA/1% Triton X-100/1 mM DTT/1 mM PMSF/1×PICS). The homogenate was centrifuged at 12,000g for 10 min, and the supernatant was incubated with SR antibody for 2 h at 4°C. Protein A/G-agarose beads were added to the mixture for 1 h at 4°C and washed with immunoprecipitation buffer (×3). Bound proteins were analyzed by Western blot with GRIP antibody.

Immunocytochemistry

Mixed glial culture cells were prepared and grown on poly-L-lysine (500 µg/mL)-coated glass coverslips, fixed with 4% paraformaldehyde in PBS for 5 min, partially trypsinized (only for anti-SR and anti-GRIP staining), permeabilized with 0.5% Triton X-100–PBS for 10 min, and blocked with 10% FBS–PBS for 10 min. Primary antibody incubations were conducted at 4°C overnight in 1% BSA–PBS. Mouse anti-glial fibrillary acidic protein (GFAP) (Research Diagnostics, Flanders, NJ) and rabbit anti-GluR2/3 (Chemicon) were used at 1 and 3 mg/mL, respectively. Rabbit anti-SR and anti-GRIP antibody were used at 1:100 and 1:200 dilutions, respectively. Rabbit secondary antibodies, FITC-conjugated anti-mouse, and Texas red-conjugated anti-rabbit (Jackson ImmunoResearch) were used at a 1:125 dilution for 1 h at 37°C. Cells were washed for 10 min (three times) with BSA–PBS after primary and secondary incubation and visualized with a confocal microscope.

D-Serine Synthesis Assay

SR and SR(V339G) cDNAs, subcloned into pTracer-CMV vector, were transfected into C6 glioma cells. After transfection (36 h), the cells were homogenized and dialyzed. Cell lysates were incubated with 1 mM L-serine and 0.5 µM pyridoxal 5' phosphate at 37°C for 3 h. This reaction was performed three times. The reaction was stopped by adding trichloroacetic acid (5% final concentration), and the amino acids were ether-extracted. Samples were dried to completion and analyzed on HPLC (8).

GRIP PDZ-6 Recombinant Adenovirus Generation

GRIP PDZ-6 cDNA was subcloned into pADTrack-CMV vector. The recombinant virus expressing GRIP PDZ-6 protein was generated and amplified following the protocol of the AdEasy Adenovirus system (Quantum Technologies, Montreal).

Adenovirus Infection

Mixed glial cells were split equally and grown to 70% confluency in DMEM with 10% FBS, 5% glutamate, and 100 units of penicillin–streptomycin at 37°C in 5% CO₂ atmosphere. The cells were infected for 36 h with GFP or GRIP–PDZ6 recombinant adenovirus. The media were replaced with 0.1×

Basal Media Eagle containing AMPA (1 mM) and 6-nitro-7-sulfamoyl-benzo(F)quinoxaline-2,3-dione (NBQX) (2 mM) or PBS, as control. Incubation with the appropriate drug was conducted for 1 h at 37°C in 5% CO₂ atmosphere. Media were removed, and the cells were harvested for HPLC analysis. The experiment was replicated four times.

Mouse Adenoviral Infection and BrdUrd Labeling

At postnatal day 8 (P8), mice were injected in the brain with 10 µL of WT adenovirus or GRIP-PDZ-6 adenovirus. Mice were injected i.p. with 25 mL of 10 µg/mL BrdUrd (Roche Molecular Biochemicals) dissolved in saline solution, 0.007 M NaOH, and 0.9% NaCl. At P11, mice cerebella were removed to assay D-serine by HPLC, and the brains, perfused with 4% paraformaldehyde, were sectioned to 10-µm slices. The sections were washed with PBS for 5 min (three times), treated with 2 M HCl for 30 min at room temperature (RT), and neutralized with 0.1 M NaB₂O₇ for 10 min at RT. Sections were washed with PBS for 5 min (three times) and blocked with 3% BSA, 1% goat serum, and 0.3% Triton X-100 in PBS for 2 h at RT. Brain sections were stained with 1:100 BrdUrd antibody (Becton Dickinson) overnight at 4°C and washed with PBS for 5 min (three times). Anti-mouse secondary were added to sections for 1 h at RT and washed with PBS for 5 min (four times). The experiment was replicated three times.

Granule Cell Migration Assay

The granule cell migration assay was performed as described (6). The cerebellar slices (800 µm), sectioned sagittally, were placed in ACSF solution (125 mM NaCl/5 mM KCl/2 mM CaCl₂/1 mM MgCl₂/24 mM glucose/10 mM Hepes) and were preincubated in six-well plates with appropriate drugs or D-amino acid oxidase (DAO) (5 µg per well; Sigma-Aldrich) for 30 min. The slices were washed with ACSF three times and treated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (10 µg/mL, Molecular Probes) for 15 min. The cerebellar slices were washed (three times) and postincubated in appropriate conditions. The migration reaction was conducted in a 37°C, 5% CO₂ incubator for 4 h and was stopped by adding 4% paraformaldehyde. The cerebellar slices were cut sagittally into 200-µm slices, visualized by using a fluorescence microscope, and the migration distance was determined by using OPENLAB software (Improvision, Lexington, MA). Drug (Sigma) concentrations used were 10 µM D-serine, 10 µM AMPA, 100 µM NBQX, 50 µM phenazine ethosulfate (Et-Phen), 50 µM phenazine methosulfate (Met-Phen), and 100 µM phenazine (Phen).

Explant Culture Preparation

Cultures were prepared as described (9). P1–P3 mouse cerebella were isolated in Hanks' balanced salt. Cerebella were freed from meninges and sag-

itally sliced into 200- to 400- μ m slices. The cerebellar cortices were isolated and sectioned into rectangular pieces. These pieces were then placed onto a poly-L-lysine/laminin-coated glass coverslip (\approx 5–10 explants per coverslip) and allowed to settle for 2–4 h. For solution bathing the explants were changed to minimum essential media (Invitrogen) supplemented with 30 mM glucose, 10% FCS, 90 units/mL penicillin, 90 μ g/mL streptomycin, and 1.8 mM glutamine.

Granule Cell Migration on SR-Transfected HEK-293 Cells

The explant cultures were placed on top of HEK-293 cells transfected with SR(WT), SR(K56G), and empty vector. HEK-293 cells were grown on poly-D-lysine-coated glass coverslips and cultured in 37°C, 5% CO₂ incubator for 2 days. Cultures were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100–PBS for 10 min, and blocked with 10% FBS–PBS for 10 min. Rabbit anti-NeuN antibody (1:1,000; Chemicon) diluted in 1% BSA–PBS was incubated with cultures at 4°C overnight. Rabbit secondary antibodies (Texas red-conjugated anti-rabbit) were used at 1:125 dilution for 1 h at 37°C. Explants were washed for 10 min (three times) with BSA–PBS after primary and secondary incubations. Granule cell migration was visualized and measured with a confocal microscope.

Calcium Imaging

Calcium imaging of spontaneous calcium transients was performed on cerebellar explant cultures after 2–3 days *in vitro*. The explants were loaded with calcium indicator Fura-2AM (Molecular Probes) by incubating in DMEM containing 2.5 μ M Fura-2AM for 15–30 min at 37°C. The coverslips were washed (four times) with fresh DMEM and incubated at 37°C for 30 min to allow for deesterification of the dye. The coverslips were then mounted onto an Olympus BX51WI microscope stage and continuously perfused with ACSF at RT. Et-Phen stock solution was made fresh in ACSF. Images were obtained by using a \times 60 LUMPlanFl objective (Olympus, Melville, NY, numerical aperture 0.90), Fura filter set (Chroma Technology, Rockingham, VT), and a charge-coupled device digital camera (C4749-98, Hamamatsu, Middlesex, NJ). Fluorescence images were obtained only for the 380-nm excitation wavelength to minimize UV exposure. Acquisition rates varied from 0.5 to 0.16 Hz, and exposure times ranged from 20 to 30 ms. Fluorescence images were analyzed by using OPENLAB software. Fluorescence data were normalized to baseline Fura 380 fluorescence levels and corrected for background. To ensure that fluorescence changes observed were not influenced by potential dye quenching, some explants were loaded with the calcium indicator Fluo-3AM (Molecular Probes) exactly as described for Fura-2AM, and a standard FITC filter cube set was used to acquire images. Data shown are with Fura-2AM.

Results

SR and GRIP Binding Interactions and Colocalization

Yeast two-hybrid analysis of full-length SR identifies GRIP containing PDZ domains 4, 5, and 6 as its interacting protein (Figure 23–1A), and SR binds selectively to PDZ-6 but not PDZ-4 or PDZ-5 (Figure 23–1B). PDZ domains interact with the three carboxyl-terminal amino acids of the partner proteins with a well characterized consensus sequence (10). The carboxyl-terminal portion of SR, -V-S-V, fits the PDZ domain binding ligand consensus. Yeast two-hybrid analysis of various portions of SR reveals that GRIP binds solely to the extreme carboxyl-terminal portion of SR (Figure 23–1C). Mutation to glycine of the carboxyl-terminal valine (V339) of SR abolishes interactions with GRIP (Figure 23–1D). Thus, like other interactors with PDZ domains, SR binds to GRIP via its carboxyl terminus. GST-tagged SR, transfected into HEK-293 cells, binds to endogenous GRIP, whereas GST-tagged SR(V339G) does not bind GRIP (Figure 23–1E). These interactions also occur *in vivo*, as GRIP and SR coimmunoprecipitate from mouse brain extracts.

For GRIP, AMPA receptors, and SR to interact, they should exist in the same cells. AMPA receptors have been demonstrated in astrocytes biochemically and functionally, and GRIP has been identified in glia (11, 12). Our immunohistochemical staining of mouse brain and primary cultures of mixed glia confirms GRIP's localization to astrocytes, whose identity is verified by staining with GFAP (Figure 23–1F, G). Primary astrocytic cultures stain for the AMPA receptor subtypes GluR2 and GluR3 as well as SR. Western blot analysis also reveals coexpression of GRIP, SR, GluR2/3, and GFAP in astrocytes (Figure 23–1G).

AMPA Receptor Activation Stimulates SR and Releases D-Serine via GRIP

We used C6 glioma cells, a continuous cell line of astrocytes, to examine the influence of GRIP on SR activity. We transfected C6 cells, which contain endogenous GRIP, either with SR(WT) or SR(V339G) and monitored conversion of L-serine to D-serine in the transfected cell lysates (Figure 23–2A). D-Serine formation is reduced 65% in cells transfected with SR(V339G), suggesting that the enzyme activity depends on interactions of SR with GRIP.

To determine whether GRIP's known interactions with AMPA receptors regulate SR, we treated primary glial cultures with AMPA (Figure 23–2B). In the absence of AMPA stimulation, levels of D-serine are ≈ 50 -fold higher in the medium than in the cells, suggesting an ongoing release process. AMPA treatment triples the release of D-serine into the medium. Treatment with the AMPA receptor antagonist NBQX markedly reduces these levels, indicating

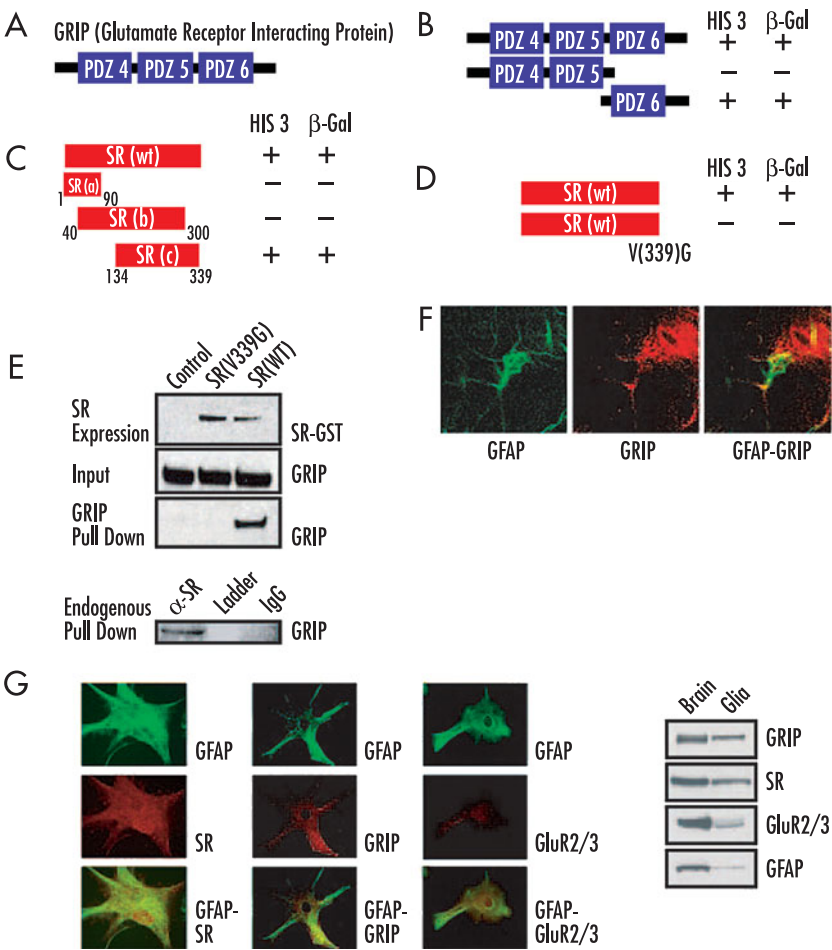


FIGURE 23-1. GRIP binds SR in yeast two-hybrid analysis and by immunoprecipitation.

(A) Of one million clones screened from rat hippocampus and cortex cDNA library, two clones were both His- and β -gal-positive. The two positive clones correspond to GRIP PDZ domains 4, 5, and 6. (B) Yeast two-hybrid analysis establishes selective binding of GRIP's PDZ domain 6 to SR. (C and D) Yeast two-hybrid analysis was used to determine which region of SR interacts with GRIP. The last amino acid of SR, Val-339, was mutated to glycine, which abolishes the interaction between SR and GRIP. (E) SR(WT) interacts with GRIP in HEK-293 cells, but SR(V339G) does not bind to GRIP. GRIP was coimmunoprecipitated from mouse brain with SR antibody. (F) Colocalization of GRIP and GFAP, a glial marker, in mouse brain. (G) Colocalization of GRIP, GluR2/3, and SR with GFAP. Primary mixed glial cultures were double-labeled by using anti-GRIP, anti-GluR2/3, anti-SR, and anti-GFAP. GRIP, SR, GluR2/3, and GRIP expression in primary glial culture is also shown by Western blot analysis.

that basal AMPA receptor activation by endogenous glutamate contributes to basal levels of D-serine.

To examine the role of GRIP in AMPA receptor-mediated D-serine release, we infected primary glial cultures with an adenovirus containing GRIP PDZ-6 with a bicistronic GFP gene. We were unable to compare effects of PDZ-6 with other PDZ domains of GRIP, because PDZ-4 and PDZ-5 bind directly to the receptor (13) and would cause interfering dominant negative effects, whereas PDZ-1, PDZ-2, PDZ-3, and PDZ-7 bind to other domains (14) that would interfere with SR studies. Virtually all cells are positive for GFP, indicating quantitative infection. With empty viral infection, D-serine levels in glial cells are only a few percent of levels in the medium. Thus, there is minimal storage of D-serine in glia with newly synthesized D-serine essentially all released into the medium (Figure 23–2C, D). NBQX treatment reduces basal D-serine levels in the medium by 80%, establishing that basal D-serine release is regulated by AMPA receptor activation (Figure 23–2C). AMPA treatment elicits release of D-serine, which is prevented by NBQX. The ability of NBQX to block the AMPA–GRIP release of D-serine fits with the notion that AMPA receptor acts via GRIP to augment D-serine release. Under all GRIP viral infection conditions, D-serine release is twice that of empty viral infection, demonstrating that GRIP regulates D-serine formation and release in physiologic glial preparations. D-Serine levels within glial cells are also markedly enhanced by GRIP viral infection with similar levels in control, AMPA-treated, NBQX-treated, and AMPA/NBQX-treated cells (Figure 23–2D). In the cells with empty viral infection, only AMPA stimulation provides detectable levels of D-serine. Presumably, activation of SR by infected GRIP augments intracellular D-serine levels to the maximal amounts that can be maintained even in the absence of AMPA treatment. Thus, AMPA receptor activation is crucial for D-serine release, and GRIP's binding to SR physiologically activates D-serine synthesis.

Viral Infection With GRIP Stimulates Neuronal Migration

Neuronal migration during development occurs along the processes of radial glia, which is best exemplified in the cerebellum. Migration of cerebellar granule cells from the EGL to the IGL uses Bergmann glia as a scaffold and is blocked by antagonists of the site of NMDA receptors that binds D-serine/glycine (7). We previously showed that SR is localized to Bergmann glia (5). To test the physiologic significance of SR and GRIP interactions, we explored granule cell migration along Bergmann glia. We infected P8 mice with WT and GRIP PDZ-6 adenovirus, which preferentially infects glial cells. Three days after viral infection, D-serine levels in GRIP adenovirus-infected cerebellum were twice those of WT adenovirus-infected cerebellum (Figure 23–3A).

We labeled granule cells with BrdUrd to observe the influence of GRIP-SR interactions on granule cell migration (Figure 23–3B, C). In GRIP adenovirus-infected mice, the proportion of labeled cells is markedly reduced in EGL and comparably increased in the molecular layer (ML) and IGL, consistent with migration from EGL toward IGL.

Granule Cell Migration in Slices Requires D-Serine and Involves a Chemokinetic Mechanism

We used two strategies to demonstrate that D-serine is selectively required for cerebellar granule cell migration, one involving its degradation by DAO and selective inhibition of SR. DAO was identified by Krebs (15) as an enzyme that degrades D- but not L-amino acids. Under physiologic conditions, DAO selectively degrades D-serine (3). DAO treatment markedly reduces NMDA neurotransmission in brain cultures and slices, establishing D-serine as an endogenous ligand mediating NMDA neurotransmission (3). To ascertain a role for D-serine in granule cell migration, we treated mouse cerebellar slices at P10 with DAO under conditions in which we demonstrate major degradation of D-serine but no loss of glycine (data not shown). DAO treatment reduces granule cell migration $\approx 60\%$ (Figure 23–4A). Because DAO generates H_2O_2 that can be cytotoxic, we examined the reversibility of inhibition by treating preparations with D-serine, which restores migration. Similar effects of DAO and reversibility by D-serine are observed whether or not DAO is maintained in contact with slices during the 4 h in which granule cell migration is monitored. As an additional test of specificity, we treated slices with sodium benzoate, a DAO inhibitor (16), which reverses effects of DAO on granule cell migration.

We also lowered D-serine levels by treating slices with SR inhibitors (Figure 23–4B). In assays of catalytic activity (17), Et-Phen and Met-Phen potently inhibit the enzyme with IC_{50} values of 5 and 3 μM , respectively, whereas Phen itself fails to inhibit enzyme activity at 100 μM , as observed also by others (C. Rojas, personal communication). Et-Phen and Met-Phen profoundly inhibit granule cell migration, whereas Phen elicits only a modest effect. The effect of Et-Phen is reduced by D-serine, ensuring that the drugs' influence on migration does not involve irreversible cytotoxic actions.

Granule cell migration depends on augmented intracellular calcium primarily derived from influx in response to NMDA receptor activation (9). We imaged intracellular calcium in migrating granule cell neurons of P2 mouse cerebellar explants (Figure 23–4C). Treatment with Et-Phen markedly diminishes intracellular calcium, and this effect is reversed by removal of the drug (data not shown).

In mediating NMDA neurotransmission, D-serine is generated after activation by glutamate of AMPA receptors on astrocytes (4). Komuro and Rakic

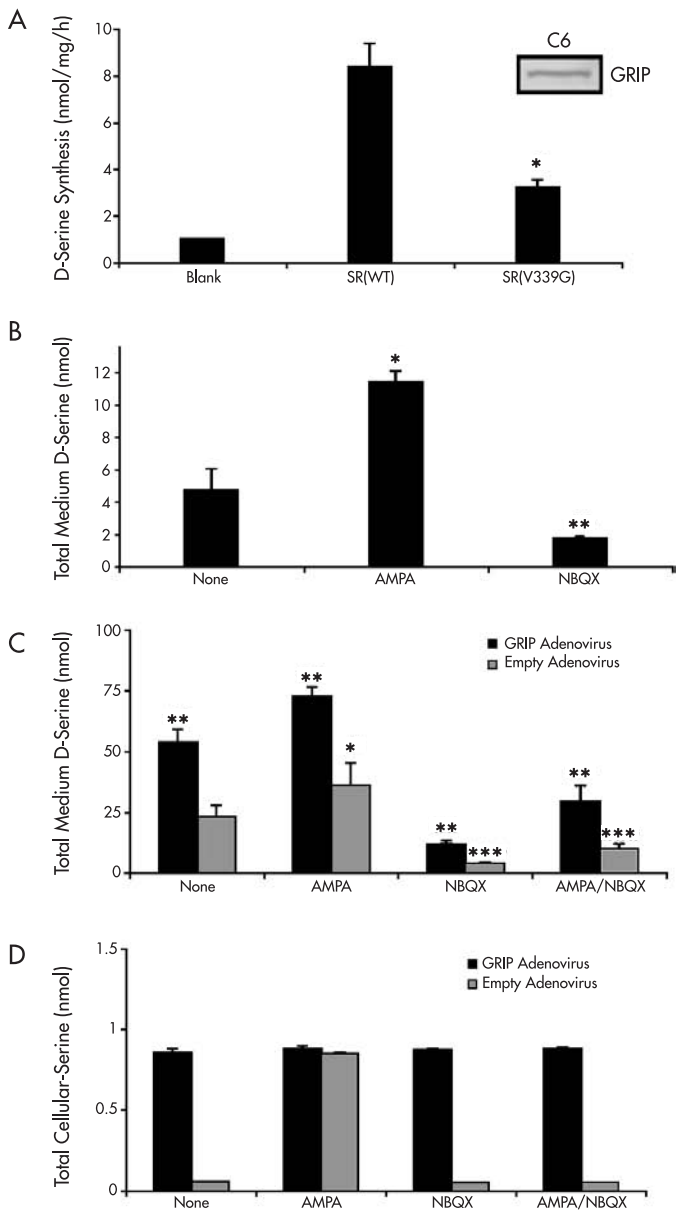


FIGURE 23-2. SR interaction with GRIP increases D-serine synthesis and release.

(A) Incubation of SR(WT)-transfected cell lysate with L-serine markedly augments D-serine synthesis, whereas incubation with cell lysate transfected with SR(V339G), which does not bind to GRIP, provides 65% less D-serine formation compared with SR(WT)-transfected cell lysate; * $P < 0.005$. (Inset) C6 glioma cells express GRIP.

FIGURE 23-2. SR interaction with GRIP increases D-serine synthesis and release (continued).

(B) AMPA stimulation elicits 2.5 times more D-serine release than control; $*P < 0.005$. D-Serine release in control samples reflects endogenous AMPA receptor activation, as it is markedly reduced by the AMPA receptor antagonist NBQX; $**P < 0.001$. (C) All GRIP virus-infected cultures display two to three times more D-serine release in the media than empty virus-infected cultures; $**P < 0.001$. AMPA stimulates and NBQX inhibits D-serine release in GRIP and empty virus-infected cultures; $*P < 0.005$ and $***P < 0.0005$. (D) With empty adenovirus, AMPA treatment increases cellular D-serine >10-fold, an effect blocked by NBQX. GRIP infection increases D-serine levels to the same values in all conditions. D-Serine levels for control, NBQX-, and AMPA/NBQX-treated cell lysates were undetectable.

(7) did not find influences of 10 μM concentrations of the AMPA antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) upon granule cell migration. We examined migration in the presence of the more potent AMPA antagonist NBQX at a higher concentration, 100 μM (Figure 23-4D) (18). Presumably, 100 μM NBQX blocks AMPA receptors more completely than the 10 μM CNQX. The specificity of NBQX is evident by its reversal with added AMPA or D-serine. Drug effects are similar whether NBQX is continuously exposed to the granule cells during the 4 h of migration or removed before migration.

How does D-serine influence granule cell migration? One possibility would be a chemokinetic influence on granule cells by increasing the activation of NMDA receptors. To examine this possibility, we evaluated granule cell migration in P2 cerebellar explants overlying HEK-293 cells transfected with SR, SR(K56G), or an empty vector (Figure 23-4E, F). In SR(K56G), the catalytic lysine is mutated to glycine, resulting in complete inactivation of the enzyme (19). Granule cell migration is markedly greater in preparations overlying SR-containing HEK-293 cells and over those with SR(K56G) or empty vector. Thus, D-serine appears to be a chemokinetic stimulus to granule cells.

Discussion

In the present study, we demonstrate a major role for GRIP in mediating activation by AMPA receptor neurotransmission of SR and D-serine release, findings consistent with the following model (Figure 23-5). Released glutamate stimulates AMPA receptors on closely adjacent protoplasmic astrocytes, causing GRIP to bind to SR, enhancing the formation and release of D-serine. Our results suggest that GRIP, AMPA receptor, and SR do not form a ternary complex because the augmentation of D-serine release associated with GRIP transfection is the same in either the presence or absence of

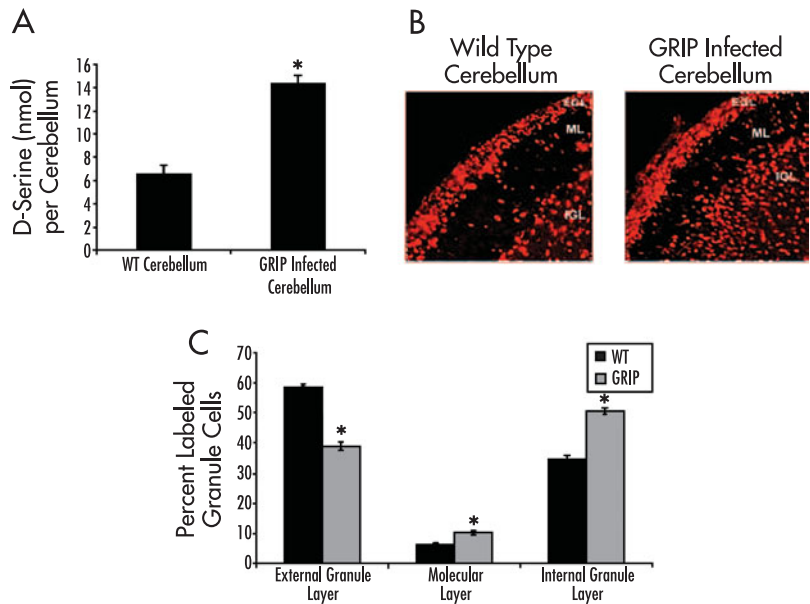


FIGURE 23-3. GRIP augments D-serine cerebellar concentrations in intact mice and accelerates cerebellar granule cell migration.

(A) GRIP adenoviral infection in P11 mice increases cerebellar D-serine levels. GRIP PDZ-6 adenovirus-infected cerebellum displays a 2-fold increase in D-serine levels compared with the empty adenoviral-infected cerebellum; $*P < 0.001$. (B) BrdUrd staining of P11 cerebellum infected with WT and GRIP adenovirus. BrdUrd-labeled granule cells are stained and can be observed in the EGL, ML, and IGL. (C) There are two times more granule cells in the ML and 30% more granule cells in the IGL for GRIP-infected cerebellum compared with WT virus-infected cerebellum; $*P < 0.001$. Granule cell density in the EGL is correspondingly reduced; $*P < 0.001$.

AMPA treatment. If GRIP enhances SR by linking AMPA receptors and SR, then SR should be stimulated by GRIP much more in the presence than in the absence of AMPA receptor activation. AMPA receptor activation triggers phosphorylation of the receptor at Ser-880, causing dissociation of GRIP (20). Such dissociated GRIP is presumably responsible for the physiological activation of SR.

Activation of SR in our experiments led to much higher levels of D-serine in the media than the cells. This finding suggests some link between SR formation of D-serine and its release. Previously, we showed that AMPA receptor activation releases [^3H]D-serine from type II astrocytes labeled by the accumulation of the [^3H]amino acid (4). It is not clear whether accumulated [^3H]D-serine in those studies labeled endogenous stores with AMPA-induced release coupled to augmented synthesis. We do not know how

D-serine is physiologically released, whether by a reversed transport mechanism or a vesicular process.

Calcium can stimulate recombinant SR purified from *Escherichia coli* (21), an action that may reflect endogenous regulation of SR by magnesium and ATP (17). We have examined the influence of calcium (0.1 mM) on SR activity in the presence of GRIP, but did not detect any change in catalytic activity (data not shown).

The regulation of SR by GRIP and its influence on D-serine release may have clinical implications. In vascular stroke, large amounts of glutamate are released and an impact on NMDA receptors is thought to be pathophysiologic, because drugs that block the glycine/D-serine site as well as the glutamate site of NMDA receptors reverse stroke damage (22). Conceivably, drugs that block the binding of GRIP to SR may also be therapeutic. Regulation of D-serine formation and release by GRIP may also be relevant to psychiatric disorders. Multiple recent studies have linked the gene for DAO to both schizophrenia and bipolar illness (23, 24). The gene for a protein designated G72, which augments DAO activity (23), is also implicated in these two psychiatric disorders. Additional evidence relating D-serine to schizophrenia includes the psychotomimetic effects of phencyclidine and other drugs that block NMDA receptors (25). The psychotomimetic actions of phencyclidine resemble schizophrenic symptoms more than those elicited by any other psychotogen. Additionally, oral administration of D-serine, D-cycloserine, and glycine alleviates schizophrenic symptoms in patients (26, 27). Thus, drugs that facilitate D-serine release by influencing interactions with GRIP might have therapeutic utility in schizophrenia.

We have also established a major physiologic role for D-serine and AMPA receptor–GRIP–SR interactions in neuronal migration. Thus, granule cell migration along Bergmann glia is blocked by degradation of D-serine or SR inhibition. The migration appears to involve GRIP influences on SR, because GRIP viral infection of intact mice augments granule cell migration. D-Serine appears to influence granule cell migration in a chemokinetic mechanism, as migration in cerebellar explants is augmented by the application of HEK-293 cells transfected with active SR but not with catalytically inactive SR. Although the D-serine that activates granule cells derives from Bergmann glia, we don't know the source of the requisite glutamate that has been suggested to arise from parallel fibers of granule cells that have already reached the IGL (28).

D-Serine may play different roles in the adult than in development, which may be mirrored in distinctive localizations at different ages. In most parts of the brain, D-serine's histochemical localizations match those of NMDA receptors much more closely than do those of glycine, consistent with a role for D-serine as the principal endogenous agonist for NMDA receptors, especially in the hippocampus where selective degradation of D-serine pro-

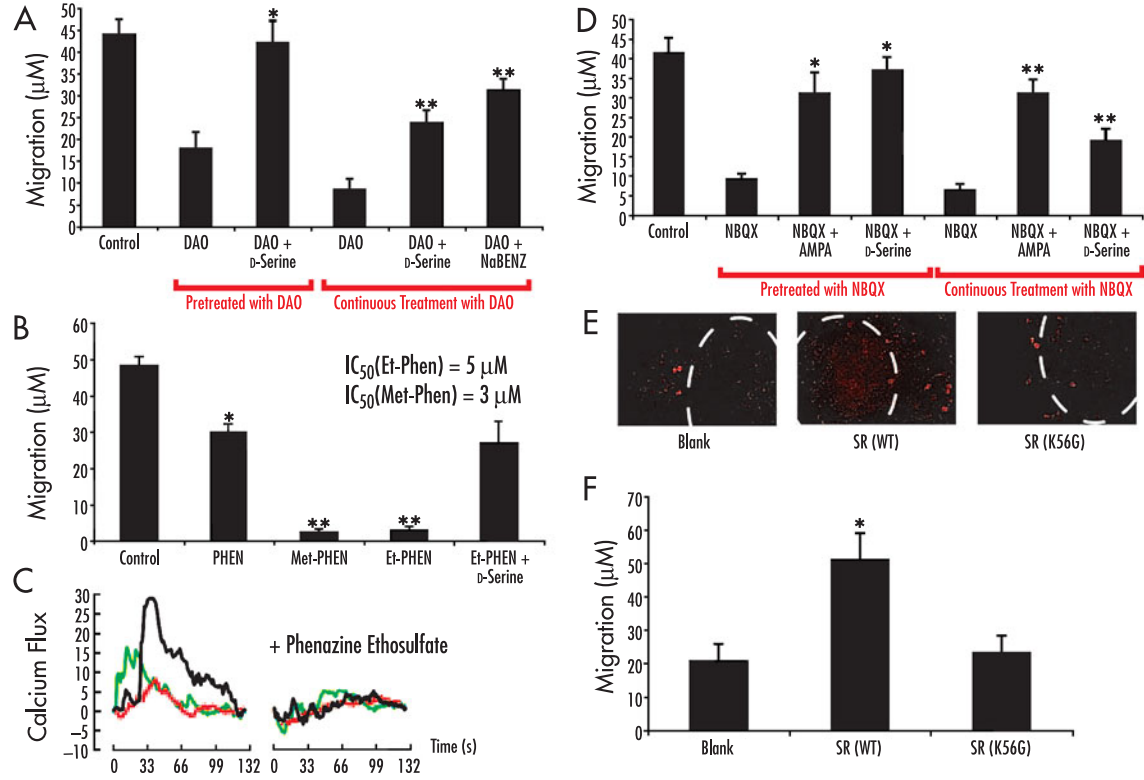


FIGURE 23–4. D-Serine increases neuronal migration.

(A) DAO and SR inhibitors decrease granule cell migration. Cerebellar slices were pretreated with DAO. When DAO is removed during the migration period, D-serine increases the granule cell migration distance by 2.5 times that of untreated slices; $*P < 0.005$. Similar results were obtained for continuous treatment with DAO ($**P < 0.005$) compared with DAO alone. Thus, D-serine or sodium benzoate (NaBENZ) rescue the granule cell migration from DAO's inhibitory effect. (B) Met-Phen and Et-Phen, inhibitors of SR block granule cell migration are shown; $**P < 0.005$. Phen inhibits activity of SR only very weakly and exerts modest inhibitory effects on granule cell migration ($*P < 0.05$) compared with control. Et-Phen's effects on granule cell migration are reversed by adding D-serine. (C) Inhibiting SR blocks granule cell calcium transients. Representative traces of spontaneous calcium transients in granule cells (3 days *in vitro*) that are inhibited by Et-Phen are shown. (D) AMPA receptor blockade by NBQX prevents granule cell migration. When NBQX was removed, granule cell migration could be rescued by adding AMPA or D-serine to the media ($*P < 0.005$) compared with NBQX alone. Similar results were obtained with continuous treatment with NBQX during the migration period ($**P < 0.005$) compared with NBQX alone. (E) D-Serine increases granule cell migration in cerebellar explant cultures. Granule cells on HEK-293 cells transfected with empty plasmid and SR(K56G), which does not bind to pyridoxal 5' phosphate, do not migrate far from the cerebellar explant, whose border is shown by a dashed line. In SR(WT)-transfected condition, granule cells migrate further than control or SR(K56G) cells. Granule cells were stained with NeuN, a neuron specific marker. (F) Granule cells ($n=30$) with SR(WT) transfection migrate more than twice the distance of control and SR(K56G) mutant preparations ($*P < 0.005$).

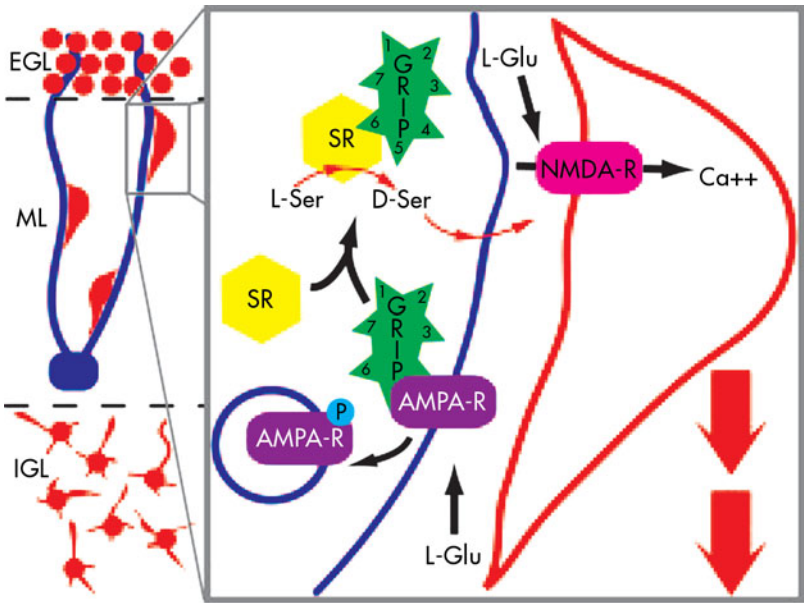


FIGURE 23-5. Model for D-serine and glutamate regulation of cerebellar granule cell migration.

Glutamate activates AMPA receptors (AMPA-R) on Bergmann glia, leading to receptor phosphorylation and dissociation of receptor-bound GRIP, which then binds to SR. GRIP activates SR with release of newly formed D-serine to join glutamate in stimulating NMDA receptor (NMDA-R) on granule cells, releasing intracellular calcium and facilitating migration from the EGL to the IGL.

foundly reduces NMDA neurotransmission (3). By contrast, in the adult cerebellum D-serine levels are very low, and localizations of glycine match those of NMDA receptors better than D-serine, suggesting that in the cerebellum glycine may be the principal endogenous agonist in the adult (29). In the neonatal cerebellum, however, D-serine levels are high, peaking at the time of granule cell migration, which suggests that the principal role of D-serine in the developing cerebellum is to serve as a coagonist for NMDA receptor-dependent granule cell migration.

Although multiple investigations support a role for NMDA receptors in neuronal migration (6, 7, 9, 30, 31, 32, 33), a recent study (34) reported that NMDA receptor subtype I-deficient cerebellar granule cells can attain normal localization in intact mice. However, that study did not examine whether NMDA receptors influenced the rate of migration, as implied by our own and other studies.

Developing neurons do not make conventional synaptic connections, so one might not anticipate regulation of migration to involve neurotransmit-

ters. Our findings, together with the earlier studies of Komuro and Rakic (6, 7, 9), establish a major role for glutamate and D-serine in migration, which does not use conventional synaptic transmission but instead uses glial release of D-serine as a neuromodulator. Our evidence that D-serine acts as a chemokinetic stimulant for granule cells reflects a novel action for neurotransmitter/neuromodulator substances.

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Part IX

NEURAL MESSENGERS OF CELL LIFE AND DEATH

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COMMENTARY

Beyond Neurotransmitters

Samuel Barondes

Anyone who takes a course in biochemistry learns about cytochrome *c*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and bilirubin, three molecules that are major players in the next group of papers. But most neuroscientists and psychiatrists tend to think of the first two as boring drones in the metabolic scheme that converts food into energy, and of bilirubin as an excretory product, molecular junk. It must therefore come as a surprise to you that the final research studies in this volume are about these three substances—substances that one does not immediately associate with Solomon Snyder's groundbreaking work on neurotransmitters. It will also surprise you that the theme that unites these three papers is not even a specific property of the nervous system but, instead, the general process of cell suicide called apoptosis. Yet, as I will explain, these studies did, indeed, grow out of Sol's interest in the details of neuronal signaling, and the discoveries he has made about apoptosis have implications for the treatment of brain diseases.

The work on cytochrome *c*, a protein that participates in oxidative phosphorylation, came about because it also happens to bind to another protein, the inositol trisphosphate (InsP₃) receptor, that is a component of one of Sol's favorite systems of neurotransmission. Triggered by familiar neurotransmitters such as serotonin, this system makes use of a second messenger called InsP₃ (or IP₃), which binds to the InsP₃ receptor as explained earlier in this volume. This binding sets in motion other reactions that are part of a complex signaling process. One of these reactions is the release of

calcium ions into the cytoplasm of the nerve cell, which, in turn, has other effects.

The most extreme effect of this calcium release, which may occur in certain developmental or pathological situations, is the triggering of apoptosis. This type of cell death is strikingly different from necrosis, in which traumatized cells rupture and call forth an inflammatory response. Apoptosis, in contrast, is a controlled process of cell death that keeps the contents of the dying cell sequestered and does not cause inflammation, making possible selective elimination of cells without much disruption of the surrounding tissue. Such quiet and orderly suicide plays an important constructive role in the sculpting of circuits during normal brain maturation, as well as a destructive role in neurodegenerative diseases.

In the paper by Boehning et al. (1; Chapter 24 in this volume), the known participation of intracellular calcium in apoptosis takes on a new twist. Evidence is presented that elevated intracellular calcium, whatever its cause, releases cytochrome *c* from its mitochondrial home, which leads to its binding to the InsP_3 receptor. This unanticipated interaction between two proteins that we tend to think of in very different contexts has profound effects on the function of the InsP_3 receptor, causing a more sustained release that favors apoptosis.

The paper on GAPDH by Hara et al. (2; Chapter 26) also derives from Sol's interest in neurotransmitters. In this case it involves another of his favorite molecules, nitric oxide (NO), a gas whose role in neurotransmission was described in the paper reprinted earlier in this volume as Chapter 18 (3). NO is made by an enzyme, nitric oxide synthase, that is activated by various signaling mechanisms. This paper shows that one of these signaling mechanisms can also give rise to apoptosis.

As with the InsP_3 receptor, this mechanism involves a novel interaction between two proteins. One is GAPDH, which NO can modify through the process called S-nitrosylation. The other is a protein called Siah1 that specifically binds to nitrosylated GAPDH. This odd couple—a complex made up of nitrosylated GAPDH and Siah1—then enters the nucleus of the cell, where it catalyzes the cleavage of certain nuclear molecules in the course of apoptosis. Of particular interest to neuroscientists is the evidence presented that the neuronal cell death following excessive stimulation of glutamate receptors involves activation of nitric oxide synthase, S-nitrosylation of GAPDH, and interaction of the latter with Siah1.

The paper by Sedlak and Snyder in this group (4; Chapter 25), about an unexpected biological effect of bilirubin, was stimulated by Sol's research on another gas that participates in neurotransmission. In this case the gas is carbon monoxide (CO), which is made by an enzyme called heme oxygenase. This enzyme works by catalyzing the reaction of heme with oxygen to give

rise to two products: CO and biliverdin, the immediate precursor of bilirubin. It is because of his interest in a specific heme oxygenase that makes CO in neurons that Sol began paying attention to bilirubin.

Once this interest was kindled, Sol became curious about the reasons that biliverdin is converted to bilirubin. As far as he was concerned, the process might as well have stopped with the formation of CO, the product he was interested in, and the excretion of biliverdin into the bile. This, indeed, is just what happens in the livers of birds, reptiles, and amphibians, which excrete biliverdin as the product of heme degradation. So why, he asked, do mammals bother to convert biliverdin to bilirubin, and at the cost of molecular energy? Might the bilirubin be more than just molecular junk? Might it actually be doing something useful?

The paper by Sedlak and Snyder presents experimental and clinical evidence that bilirubin does, indeed, play a useful role by acting as an antioxidant that protects cells from dying. Furthermore, when bilirubin reacts with potentially dangerous oxidants it is converted back to biliverdin; and the bilirubin can be regenerated by biliverdin reductase, the enzyme that made it in the first place. In this way bilirubin can keep sucking up harmful oxidants.

The work with bilirubin is particularly interesting because it immediately suggests some therapeutic applications in diseases in which release of oxidants plays a role in cell death. As Sedlak and Snyder point out, 99% of the bilirubin in the human bloodstream cannot act as a cellular antioxidant, because it is bound to plasma proteins. But what if drugs were found that could release some of it into a free form that could get into cells? Might such drugs have useful therapeutic properties in neurodegenerative disorders, and in the immediate aftermath of a stroke?

These are the types of questions we have come to expect from Sol Snyder. Throughout his exceptional career he has kept one eye on neuroscience and the other on patients. So no one should be surprised if his work on bilirubin leads to the development of new medications. And no one should be surprised if his discoveries about GAPDH and cytochrome *c* also turn out to have practical applications.

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CHAPTER 24

Cytochrome *c* Binds to Inositol 1,4,5 Trisphosphate Receptors, Amplifying Calcium-Dependent Apoptosis

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Mitochondrial cytochrome *c* release and inositol 1,4,5-trisphosphate receptor (InsP₃R)-mediated calcium release from the endoplasmic reticulum mediate apoptosis in response to specific stimuli. Here we show that cytochrome *c* binds to the InsP₃R during apoptosis. Addition of 1 nM cytochrome *c* blocks calcium-dependent inhibition of InsP₃R function. Early in apoptosis, cytochrome *c* translocates to the endoplasmic reticulum where it selectively binds InsP₃R, resulting in sustained, oscillatory cytosolic calcium increases. These calcium events are linked to the coordinate release

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of cytochrome *c* from all mitochondria. Our findings identify a feed-forward mechanism whereby early cytochrome *c* release increases InsP₃R function, resulting in augmented cytochrome *c* release that amplifies the apoptotic signal.

Calcium signaling mediates many cellular functions, including programmed cell death through influences on free radical production, organelle disruption, and proteolytic enzyme activation (1, 2). Increases of cytosolic calcium during cell death can arise from several sources, including damage to external membranes, hyperactivation of ligand gated ion channels, or release from calcium stores in the endoplasmic reticulum (3, 4). During cell death, mitochondrial calcium overload activates the permeability transition pore (PTP), resulting in transient mitochondrial depolarization and decreased ATP production. PTP gating may also cause release of mitochondrial proteins that activate apoptotic pathways (5). One of these proteins, cytochrome *c*, is a small heme-containing enzyme of the electron transport chain. In many models of apoptosis, cytochrome *c* is released from the intermembrane space of mitochondria into the cytosol (5). Cytochrome *c* release can be rapid and coordinated between all mitochondria through an unknown mechanism (6). Released cytochrome *c* then binds to apoptotic protease activating factor-1 (APAF-1), which recruits and activates caspase-9 to form the apoptosome (5). Caspase-9 activation results in the cleavage and activation of caspases-3 and -7, initiating a proteolytic cascade that ultimately results in cell death.

Recent research reveals a privileged communication between mitochondria and endoplasmic reticulum calcium stores (7, 8). Apoptotic stimuli such as staurosporine (STS) and ceramide elicit InsP₃R-induced increases in mitochondrial calcium, resulting in PTP-mediated decrease in mitochondrial membrane potential and release of cytochrome *c* (4).

Using a combination of yeast two-hybrid analysis and biochemical techniques, we now demonstrate the direct binding of cytochrome *c* to InsP₃R during apoptosis. As little as 1 nM cytochrome *c* abolishes calcium-mediated inhibition of InsP₃-mediated calcium release. In the early stages of apoptosis, cytochrome *c* translocates from mitochondria to endoplasmic reticulum-containing fractions, binding specifically to InsP₃R. Cytochrome *c* translocation to the endoplasmic reticulum is altered in DT40 cells that lack all three InsP₃R isoforms. Furthermore, imaging studies reveal large, sustained increases in cytosolic calcium during early apoptosis that are concomitant with InsP₃R-cytochrome *c* interactions, as revealed by fluorescence resonance energy transfer (FRET). These cytochrome *c*–InsP₃R interactions provide a molecular mechanism whereby InsP₃R and mitochondria can work in concert to execute the apoptotic cell death program.

Results

Cytochrome *c* Binds InsP₃R, Altering Function

To identify intracellular proteins that interact with InsP₃R, we conducted a yeast two-hybrid screen of a rat brain cDNA library with the type-1 InsP₃R as bait (Figure 24–1A). Cytochrome *c* was identified as one of the interacting proteins. Transformation of yeast with varying truncations of InsP₃R revealed that cytochrome *c* binds selectively to amino acids 2589–2749 (the carboxy-terminal tail) of the InsP₃R. To confirm the binding of these two proteins, we used a glutathione *S*-transferase (GST) pulldown assay in which purified cytochrome *c* was incubated with GST–InsP₃R fusion proteins (Figure 24–1B). As in the yeast two-hybrid analysis, only the C-terminal tail bound to cytochrome *c*. Full-length InsP₃R from crude cerebellum lysate and InsP₃R purified to homogeneity bound to cytochrome *c*–agarose (Figure 24–1C), indicating a direct association.

InsP₃R activity is biphasically regulated by cytosolic calcium levels. At resting calcium concentrations (~100 nM), increases in calcium augment InsP₃-induced calcium release (9, 10). In contrast, concentrations of calcium greater than ~5 μM diminish the ability of InsP₃ to release calcium, presumably representing a safety mechanism to prevent a feed-forward excessive release of calcium (9, 10). To examine the influences of cytochrome *c* on InsP₃R function, we measured calcium release from microsomal vesicles prepared from COS cells cotransfected with type-1 InsP₃R and the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA; Figure 24–1D) (11). At the optimal calcium concentration for InsP₃R activity (250 nM), concentration–response relationships for InsP₃-mediated ⁴⁵Ca²⁺ release were the same in the presence or absence of cytochrome *c*. At 1 nM cytochrome *c*, however, calcium-induced inhibition of InsP₃R calcium release was abolished (Figure 24–1E). Cytochrome *c* had no effect on SERCA activity in the absence of InsP₃ using this assay (data not shown). High concentrations of cytochrome *c* (100 nM) were functionally indistinguishable from 1 nM cytochrome *c*, whereas 100 pM cytochrome *c* had no measurable effect on channel function (data not shown).

Cytochrome *c* Binds to InsP₃R During Apoptosis

Most InsP₃Rs in cells are associated with the endoplasmic reticulum, whereas cytochrome *c* is a classic mitochondrial constituent. We hypothesized that cytochrome *c* translocates to the endoplasmic reticulum during apoptosis to interact with InsP₃R. To explore this possibility, we induced apoptosis in HeLa and PC12 cells with the broad-spectrum kinase inhibitor STS (STS) and monitored levels of cytochrome *c* by subcellular fractionation. We examined fractions containing mitochondria and heavy endoplas-

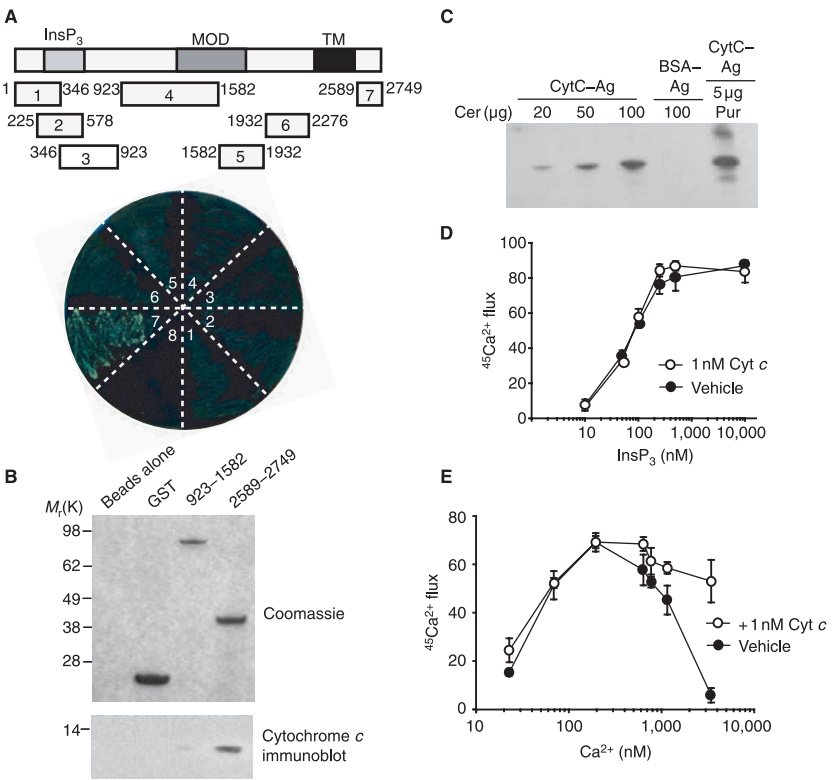


FIGURE 24-1. Cytochrome *c* interacts with InsP_3R .

(A) A schematic representation of InsP_3R . Functional domains of the InsP_3R , including the ligand-binding (InsP_3), modulatory (MOD), and transmembrane (TM) regions, are indicated. The seven regions used as bait in the yeast two-hybrid screen are also indicated below the protein and the corresponding amino acids (rat type-1 sequence). When cytochrome *c* was screened against all InsP_3R baits, only bait 7 (the C-terminal fragment of InsP_3R) supported growth on selective media. (B) *In vitro* GST fusion protein binding of purified cytochrome *c* to glutathione-Sepharose alone, GST, or 2 fragments of the InsP_3R corresponding to bait fragments 4 and 7 from the yeast two-hybrid screen. Only the C-terminal fragment (2589–2749) precipitated cytochrome *c*, as visualized by immunoblotting. (C) InsP_3R in rat cerebellar lysate and purified InsP_3R bind to cytochrome *c*-agarose. Increasing concentrations of rat cerebellar lysate and 5 μg purified InsP_3R were incubated with cytochrome *c*-agarose and washed as described. Proteins bound to beads were resolved by SDS-PAGE and visualized by immunoblotting. BSA-agarose, used as a negative control, fails to precipitate InsP_3R . (D) InsP_3 concentration-response curve in the absence or presence of 1 nM cytochrome *c*. Cytochrome *c* was added simultaneously with InsP_3 where indicated. Cytochrome *c* alone elicited no release (data not shown). The calcium concentration was 250 nM. (E) Calcium dose-response curve in the absence or presence of 1 nM cytochrome *c*. The InsP_3 concentration was 10 μM .

mic reticulum (P2), fractions containing microsomal (light) endoplasmic reticulum (P3), and the cytosolic (S3) fraction (Figure 24–2A). At 4 and 6 h after STS treatment we observed a decrease of cytochrome *c* immunoreactivity in the P2 fraction of HeLa cells that was matched by corresponding increases in the P3 fraction, with little cytochrome *c* detected in the S3 fraction. Extended exposures revealed small but detectable increases in cytochrome *c* in the S3 fraction in response to STS (data not shown). The crude microsomal fraction P3 contains light endoplasmic reticulum, but can be also contaminated by other cellular constituents. To ensure that the P3 fraction was devoid of mitochondria, we monitored the mitochondrial enzyme cytochrome *c* oxidase (CytOx), which we found exclusively in the P2 fraction at all times. Heme oxygenase-2 (HO2) is a microsomal constituent (12) that we detected in P2 and P3, but not S3, demonstrating that the S3 fraction is devoid of endoplasmic reticulum membranes. Lactate dehydrogenase (LDH), a cytoplasmic marker, is evident in S3, but not P2 and P3, with no time-dependent alterations. CytOx, HO2, and LDH immunoreactivity were also used as loading controls for each experiment.

The time-dependent decrease of cytochrome *c* in P2 and corresponding increase in P3 implies a translocation of cytochrome *c* from mitochondria to endoplasmic reticulum during apoptosis. To ascertain whether cytochrome *c* in the endoplasmic reticulum is associated with InsP₃R, we performed immunoprecipitations with an antibody to the type-1 InsP₃R (Figure 24–2B). We detected co-immunoprecipitation of the two proteins from the P3 fraction 4 h after STS treatment, with increased co-immunoprecipitation at 6 h. We did not observe co-immunoprecipitation of cytochrome *c* with SERCA, or when preimmune InsP₃R serum was used, demonstrating the specificity of this interaction. Similar experiments were conducted with the type-3 InsP₃R, providing essentially the same results (data not shown). As a result of the poor quality of available type-2 InsP₃R antibodies and the low endogenous levels of type-2 InsP₃R in most tissues (13), we have been unable to conduct experiments to monitor its interactions with cytochrome *c*. We confirmed that STS treatment induces apoptosis in our preparations by monitoring caspase-3-like catalytic activity in the S3 fraction at 4 and 6 h after STS treatment (Figure 24–2C). DNA fragmentation was also observed at later times (~12–24 h; data not shown). We performed similar experiments in HeLa cells using the physiological ligand C2-ceramide to induce apoptosis. Ceramide elicits the same effects as STS, with cytochrome *c* selectively partitioning to the P3 fraction at early times (see Supplementary Information, Figure 24–S1).

At the zero time point before STS treatment, we detected cytochrome *c* in the P3 fraction (Figure 24–2A). As the P3 fraction is devoid of mitochondrial contamination, we wondered whether the cytochrome *c* in the P3 frac-

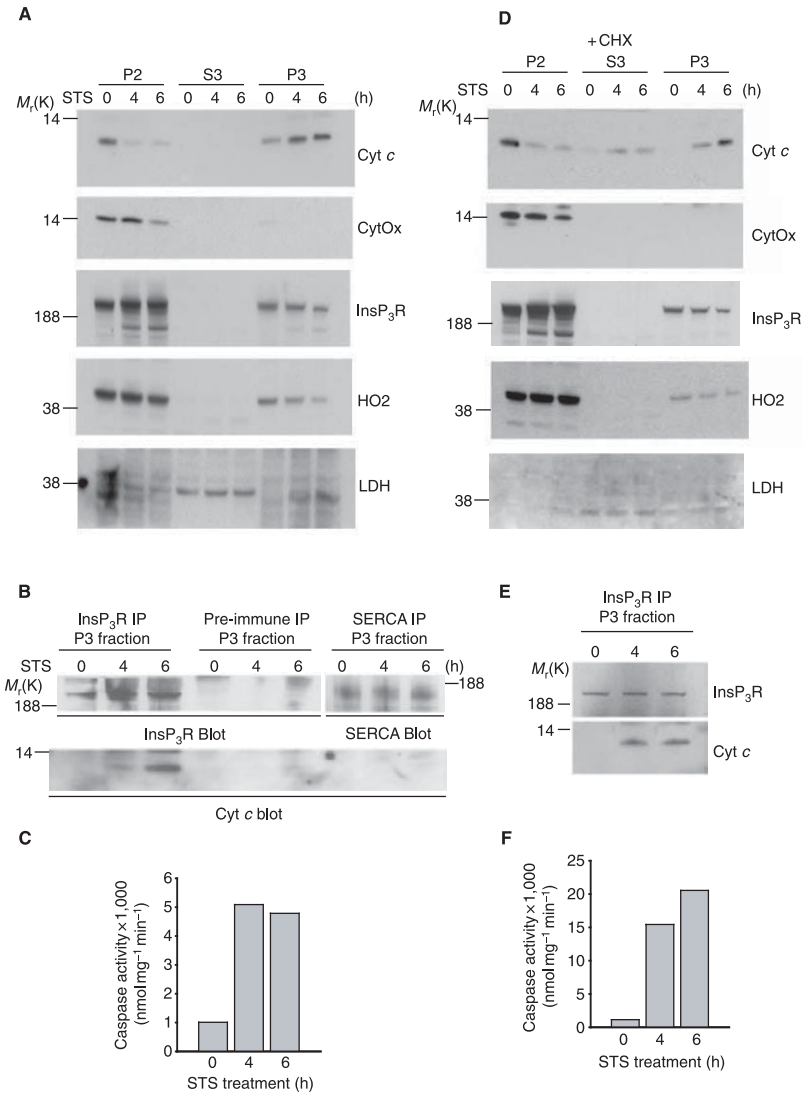


FIGURE 24-2. Cytochrome *c* translocates to the endoplasmic-reticulum-binding InsP₃R during apoptosis in HeLa cells.

(A) Cytochrome *c* translocates from the mitochondria (P2) to the endoplasmic reticulum (P3) fraction during apoptosis. Apoptosis was induced with 1 μ M STS for 0, 4, or 6 h. Cells were then harvested, fractionated, and processed. CytOx (mitochondria), HO2 (endoplasmic reticulum), and LDH (cytosol) were used to check purity of the fractions and to ensure equal protein distribution in all lanes. (B) P3 fractions (100 μ g) from STS-treated HeLa cells were immunoprecipitated with anti-InsP₃R antibody, pre-immune serum, or anti-SERCA2 antibody. Immunoprecipitates were then immunoblotted with anti-cytochrome *c*, -InsP₃R, and -SERCA antibodies. Cytochrome coprecipitates

FIGURE 24-2. Cytochrome *c* translocates to the endoplasmic-reticulum-binding InsP₃R during apoptosis in HeLa cells (*continued*).

in an STS-dependent manner only in InsP₃R immunoprecipitates. (C) The cytosol-enriched (S3) fraction was used to measure caspase-3 activity (see Methods). Panels (A)–(C) are from the same experimental treatment, and are representative of four separate determinations. (D) Inhibiting protein synthesis eliminates cytochrome *c* immunoreactivity in the P3 fraction at 0 h. This experiment was performed as in (A), except that cells were pre-incubated with 10 µg mL⁻¹ cycloheximide (CHX) for 2 h before STS treatment. Cycloheximide treatment does not interrupt cytochrome *c* translocation to the endoplasmic reticulum. Cycloheximide accelerates the appearance of cytochrome *c* in the cytosol (S3) fraction and is concurrently associated with a large increase in caspase activity relative to no CHX treatment, as shown in (F). (E) Experiment was performed as in (B), except that the P3 fraction used was from subcellular fractionation in (D). Cycloheximide does not interrupt cytochrome *c*–InsP₃R binding. (F) As was performed as in (C), except that the S3 fraction used was from the subcellular fractionation in (D). Panels (D)–(F) are from the same experimental treatment, and are representative of three separate determinations.

tion at the zero time point might represent newly synthesized protein associated with ribosomal membranes. To explore this possibility, we treated HeLa cells with the protein synthesis inhibitor cycloheximide (Figure 24–2D). Cycloheximide treatment abolished cytochrome *c* in the P3 fraction at the zero time point. This treatment also resulted in the appearance of cytochrome *c* at 4 and 6 h in the cytosolic S3 fraction, which is devoid of cytochrome *c* in the absence of cycloheximide treatment, suggesting that cycloheximide increases the susceptibility to STS-induced apoptosis in HeLa cells. This hypothesis is supported by the fourfold increase in cytosolic caspase activity in cycloheximide-treated preparations, compared with those treated only with STS (Figure 24–2C, F). In cycloheximide- and STS-treated cells, InsP₃R and cytochrome *c* also co-immunoprecipitated in an STS-dependent manner (Figure 24–2E).

To ensure that the translocation of cytochrome *c* from mitochondria to the endoplasmic reticulum is a general phenomenon, we examined STS-induced apoptosis in PC12 cells (Figure 24–3A). As in HeLa cells, PC12 cells manifest increased cytochrome *c* associated with the P3 fraction in response to STS, with a concomitant decrease in the P2 fraction. Similarly to HeLa cells, we did not detect cytochrome *c* release into the S3 fraction early in STS-induced apoptosis, and the distribution among subcellular fractions of InsP₃R, HO2 and LDH was the same in PC12 cells as in HeLa cells. CytOx immunoreactivity was not present in the S3 or P3 fractions, and endoplasmic reticulum contamination was not observed in the S3 fraction. InsP₃R and cytochrome *c* also co-immunoprecipitated from PC12 cell lysates in an STS-dependent manner (Figure 24–3B). PC12 cells demonstrated a time-

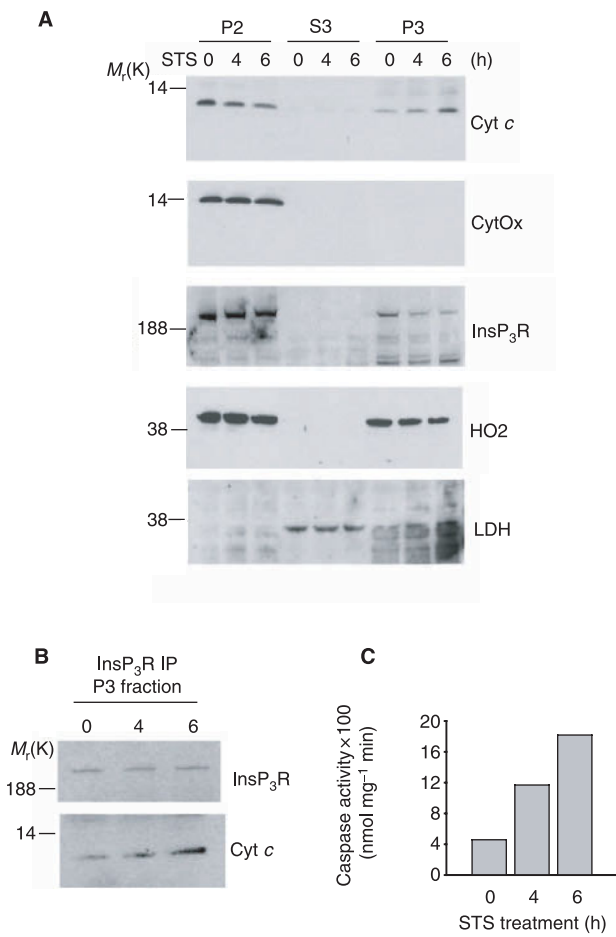


FIGURE 24–3. Cytochrome *c* translocates to the endoplasmic-reticulum-binding InsP₃R during apoptosis in PC12 cells.

(A) Cytochrome *c* translocates from the mitochondria-enriched fraction (P2) to the endoplasmic reticulum (P3) fraction during apoptosis. Experimental details were identical to Figure 24–2A, except that PC12 cells were used instead of HeLa cells. (B) Cytochrome *c* coprecipitates with InsP₃R in an STS-dependent manner. (C) Caspase-3 activity in response to STS treatment. Panels (A)–(C) are from the same experimental treatment, and are representative of five separate determinations.

dependent increase in caspase activity in cytosolic fractions (Figure 24–2C), similar to HeLa cells.

The cytochrome *c*–InsP₃R interaction suggests that during initial phases of apoptosis, cytochrome *c* is released from mitochondria to bind to InsP₃R in the closely adjacent endoplasmic reticulum. This would diminish cal-

cium-induced inhibition of InsP₃-mediated release of calcium, presumably resulting in mitochondrial and cytosolic calcium overload. As increases in caspase activity occur within the time course of cytochrome *c* translocation to the P3 fraction, we examined whether the broad-spectrum caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) affects cytochrome *c* translocation. Z-VAD-FMK completely inhibited caspase activity induced by STS in HeLa cells (Figure 24–4B). However, Z-VAD-FMK has no effect on cytochrome *c* translocation to the P3 fraction (Figure 24–4A, B), demonstrating that translocation is independent of caspase activation.

InsP₃R can be cleaved by caspases and the calcium-sensitive proteolytic enzyme, calpain, and such cleavage may participate in apoptotic cell death (14, 15). To ascertain whether cleavage of InsP₃R by these enzymes occurs during the early time phase of apoptosis that we examined, we monitored InsP₃R protein levels in HeLa cells treated with Z-VAD-FMK and the calpain inhibitor MDL-28170 (Figure 24–4C). We detected only a small amount of the reported cleavage fragment of InsP₃R (15) in response to STS, and this was not blocked by calpain inhibition (Figure 24–4C; indicated by an asterisk). Z-VAD-FMK blocked this limited degradation, consistent with caspase cleavage. Caspase-3 proteolytic activity was confirmed by monitoring cleavage of the known caspase substrate poly(ADP ribose) polymerase (PARP). We also examined the influence of caspase and calpain inhibitors in PC12 cells, exploring later as well as earlier times after STS treatment. Similarly to HeLa cells, InsP₃R levels remained constant 4 and 8 h after STS treatment, whereas PARP cleavage was observed (Figure 24–4D). At later times, we observed a progressive decrease in InsP₃R, with almost complete absence at 24 and 48 h. Z-VAD-FMK partially reduced this decline, but we could not determine whether this effect reflects inhibition of enzymatic activity cleaving InsP₃R or is secondary to prevention of apoptosis by Z-VAD-FMK. The calpain inhibitor failed to alter the decline of InsP₃R at later times. As little to no InsP₃R degradation is observed at the early times of STS-induced apoptosis, we speculate that InsP₃R function is maintained during the early stages of apoptosis and that cytochrome *c* may be influencing calcium homeostasis in response to apoptotic stimuli.

The translocation of cytochrome *c* to the P3 fraction and its binding to InsP₃R imply that endoplasmic reticulum-associated InsP₃R is responsible for the translocation. To examine this hypothesis directly, we used DT40 B lymphocytes, in which all three isoforms of the InsP₃R are deleted (16) (Figure 24–5A, B). Although these cells have been demonstrated to be fairly resistant to apoptosis (16), they still provide an excellent model for this study as we are examining the redistribution of cytochrome *c* within the population of cells. Using a physiological stimulus, we induced apoptosis by acti-

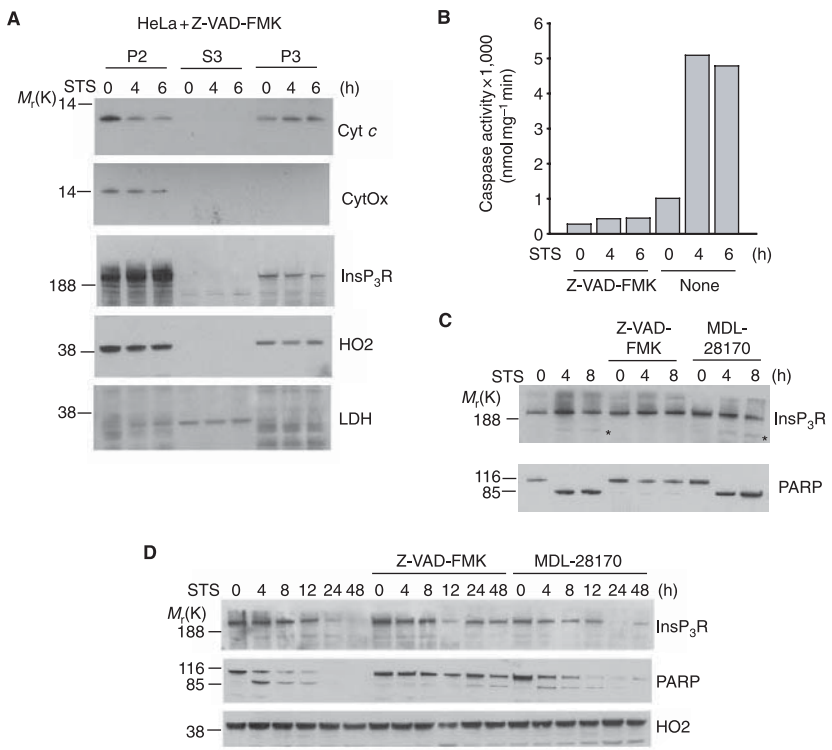


FIGURE 24-4. Translocation of cytochrome c to the endoplasmic reticulum is upstream of caspase activation.

(A) Cytochrome c translocates from the mitochondria fraction (P2) to the endoplasmic reticulum-containing (P3) fraction in the absence of caspase activity. Experimental details were identical to those described in Figure 24-2A, except that cells were pre-incubated with 50 μ M caspase inhibitor Z-VAD-FMK for 2 h before STS treatment. (B) Z-VAD-FMK blocks STS-induced increases in caspase activity. Caspase activity was measured from the cytosol-containing (S3) fraction from (A). Caspase-3 activity from Figure 24-2C is included for comparison. (C) Proteolytic cleavage of InsP₃R in HeLa cells in response to STS. Cells were pre-incubated for 2 h with 50 μ M Z-VAD-FMK or the calpain inhibitor MDL-28170 (2 μ M) before STS treatment. Cells were harvested at the indicated times after STS treatment and immunoblotted with anti-InsP₃R and anti-PARP antibodies. The reported 98K InsP₃R fragment is marked with an asterisk. (D) Proteolytic cleavage of InsP₃R in PC12 cells in response to STS. InsP₃R protein levels remain constant over the first 12 h, and progressively decrease at 24 and 48 h. Cells were treated as in (C) for the indicated times, and HO2 was used as a loading control.

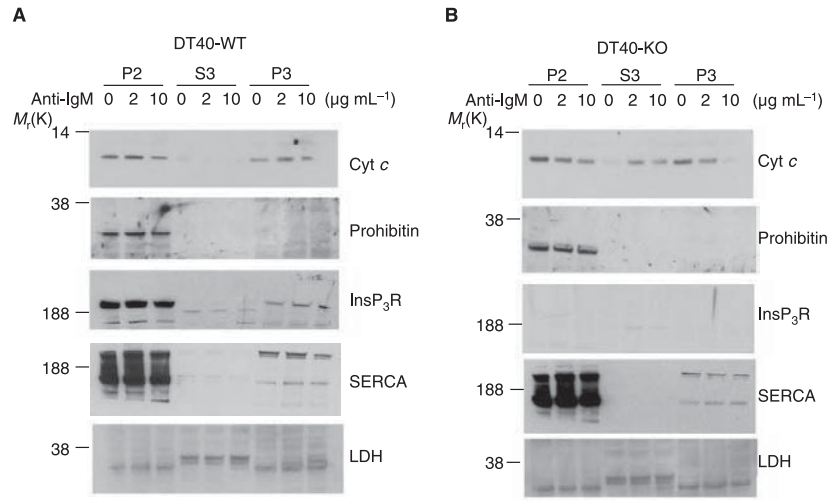


FIGURE 24-5. Cytochrome *c* translocation to endoplasmic reticulum is blocked in the absence of InsP₃R.

(A) Cytochrome *c* translocates from the mitochondria (P2) to the endoplasmic reticulum (P3) during anti-IgM induced apoptosis. DT40 cells were treated with 0, 2, or 10 $\mu\text{g mL}^{-1}$ anti-IgM for 24 h to induce apoptosis, and then fractionated. (B) Cytochrome *c* translocation in DT40 cells lacking all three isoforms of the InsP₃R. The experiments in (A) and (B) were repeated at least four times.

vating the B-cell receptor with anti-immunoglobulin M (IgM) for 24 h at concentrations of 2 or 10 $\mu\text{g mL}^{-1}$. As in HeLa and PC12 cells, anti-IgM treatment of wild-type DT40 cells elicited a decrease of cytochrome *c* in the P2 fraction and an increase in P3 fraction (Figure 24-5A). Because the CytOx antibody used in this study does not recognize the chicken isoform, we used antibodies against the avian mitochondrial protein prohibitin as a marker for mitochondria (17). Prohibitin was abundant in the P2 fraction, but not in the P3 or S3 fractions. SERCA and InsP₃R were abundant in the P2 fraction, with lower levels in the P3 fractions, whereas LDH was restricted to the S3 fraction. In DT40 cells lacking InsP₃R, anti-IgM depleted cytochrome *c* from the P2 fraction and resulted in its appearance in S3 but not P3. As in HeLa and PC12 cells, cytochrome *c* was found in the P3 fraction of untreated triple-InsP₃R knockout cells (Figure 24-5B). We could not ascertain whether this was the result of new protein synthesis because translational inhibitors also blocked anti-IgM-induced apoptosis (data not shown). Thus, in cells lacking InsP₃R, cytochrome *c* fails to translocate to the endoplasmic reticulum but instead appears in the cytoplasm, establishing that InsP₃R mediates binding of cytochrome *c* to the endoplasmic reticulum.

Cytochrome *c*–InsP₃R Binding in Intact Cells

To evaluate cytochrome *c*–InsP₃R interactions in intact cells, we used PC12 cells stably transfected with enhanced yellow fluorescent protein (EYFP)-labeled cytochrome *c*, then transiently transfected the cells with enhanced cyan fluorescent protein (ECFP) fused to the amino terminus of the InsP₃R. If cytochrome *c* and the InsP₃R come within sufficient proximity, ECFP (donor) emission at ~480 nm will be quenched and EYFP (acceptor) emission at ~535 nm will increase. Increases in the 535:480-nm ratio reflect a close association of the two proteins (less than 100 Å) (18).

We observed a gradual increase of the emission ratio over time in response to STS, beginning at ~1 h and extending through a 5-h time course (Figure 24–6A, B, C). Surface plot display of the 535:480-nm ratio in two PC12 cells demonstrates the spatial increases in InsP₃R–cytochrome *c* interactions (Figure 24–6A). We observed these increases at very early times in extranuclear compartments of the cell periphery, which is consistent with mitochondrial localization in PC12 cells (Figures 24–6A, 24–7A). This increase in the 535:480-nm ratio varied among individual cells (Figure 24–6B), demonstrating that STS induction of apoptosis is asynchronous among a population of cells. The increase in the emission ratio, averaging approximately 0.3, is comparable with values obtained for numerous other interacting proteins (19, 20). No increase was observed if STS was omitted (Figure 24–6C), or in cells where cytosolic CFP (unattached to the InsP₃R) was transfected (Figure 24–6D). These experiments establish that cytochrome *c* and InsP₃R are bound to each other during STS-induced apoptosis.

Cytochrome *c*–InsP₃R Binding and Cytosolic Calcium

To ascertain whether cytochrome *c*–InsP₃R binding mediates apoptosis-associated intracellular calcium increases, we conducted simultaneous imaging of intracellular calcium and cytochrome *c* disposition (Figure 24–7). In HeLa cells stably expressing YFP–cytochrome *c* and treated with STS, we observed gradual increases of calcium levels with an oscillation frequency of ~20 min (Figure 24–7B). At ~270 min, calcium levels reached a plateau and no further oscillations were evident. This sustained increase in cytosolic calcium continued until 480 min without further oscillations, and no releasable calcium stores were observed after treatment with 1 μM ionomycin at the 480-min timepoint (data not shown). To ensure that the observed oscillations were not caused by the destruction of the endoplasmic reticulum calcium stores by STS, we used thapsigargin at various time-points after treatment with STS, and do not see a marked decrease in thapsigargin-releasable endoplasmic reticulum calcium until 300 min, a time-point after which the oscillations are no longer evident (see Supplementary Information, Figure 24–S3). Furthermore, these oscillations are not dependent on caspase

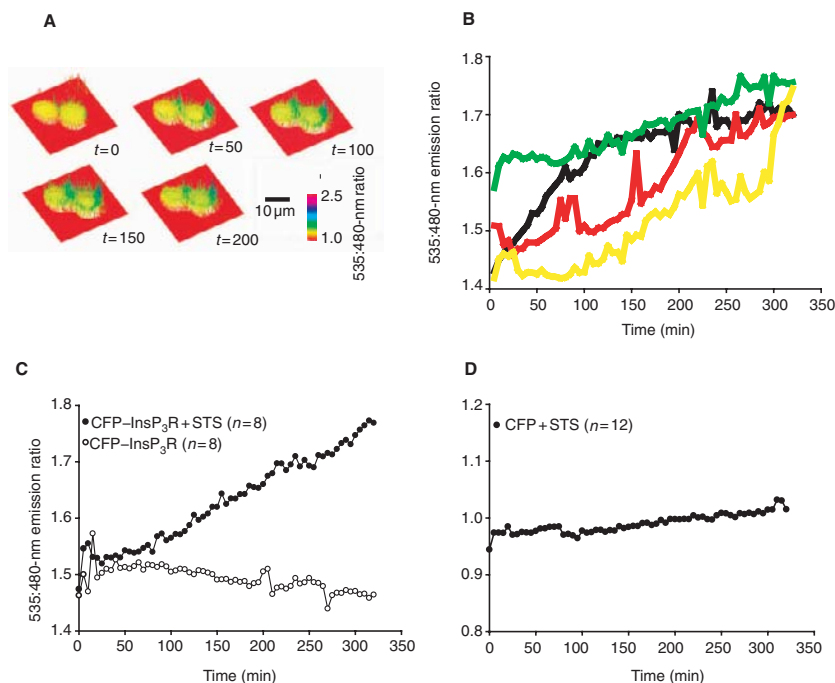


FIGURE 24-6. Cytochrome *c*-InsP₃R interaction during apoptosis demonstrated by FRET.

(A) Surface plot display of the 535:480-nm ratio in two PC12 cells stably expressing YFP-cytochrome *c* and coexpressing CFP-InsP₃R after treatment with 1 μ M STS. (B) 535:480-nm ratio of four PC12 cells stably expressing YFP-cytochrome *c* and cotransfected with CFP-InsP₃R. This plot demonstrates the heterogeneity of cytochrome *c*-InsP₃R interactions observed in response to 1 μ M STS. (C) 535:480-nm ratio of YFP-CytC PC12 cells coexpressing CFP-InsP₃R in response to 1 μ M STS or vehicle. Only cells treated with STS were positive for FRET activity. (D) 535:480-nm ratio of YFP-cytochrome *c* PC12 cells cotransfected with cytosolic CFP in response to 1 μ M STS. No change in the 535:480-nm ratio was observed.

activity, as HeLa cells pretreated with caspase inhibitors displayed identical calcium oscillations in response to STS treatment (see Supplementary Information, Figure 24-S2).

Cytochrome *c* fluorescence was initially punctate and reflects localizations in mitochondria, as established by colocalization with the mitochondrial stain MitoTracker Red (Figure 24-7A). Single-cell imaging revealed a spike of calcium immediately preceding a coordinated release of cytochrome *c* throughout the cell (Figure 24-7C). After this spike, calcium levels remained at the pre-spike level indefinitely. Averaged traces from large numbers of PC12 cells stably expressing YFP-cytochrome *c* revealed a sim-

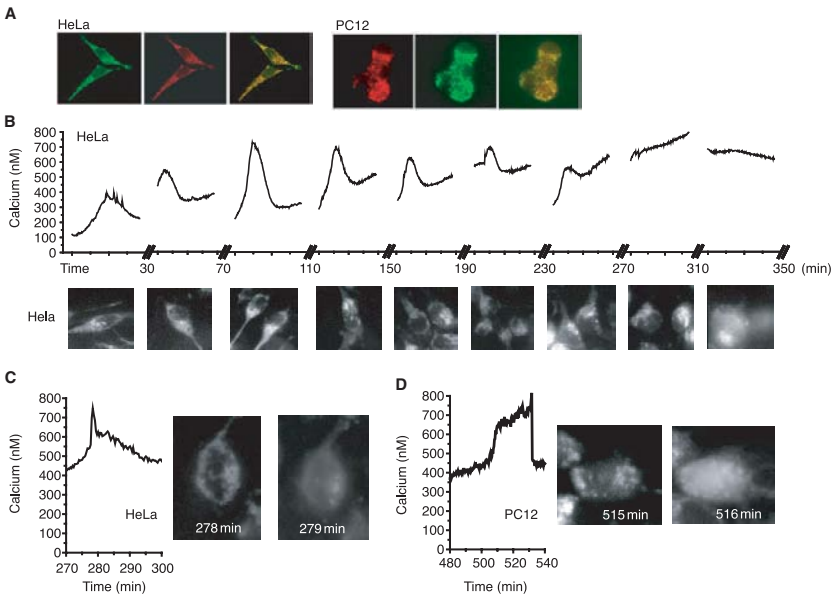


FIGURE 24-7. Changes in cytosolic calcium coincide with the coordinate release of cytochrome c from mitochondria.

(A) YFP-cytochrome c colocalizes precisely with MitoTracker Red, demonstrating that the stably expressed YFP-cytochrome c is contained within the mitochondria. (B) Fura-2 intracellular calcium measurements in response to 1 μM STS in HeLa cells stably expressing YFP-cytochrome c. Marked oscillations are observed as early as 10 min and continue until approximately 270 min. Cells displayed beneath each 30-min time course manifest the representative distribution of YFP-cytochrome c during that time course. (C) Fura-2 intracellular calcium measurements of a single HeLa cell in response to 1 μM STS over the 270-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome c into the cytosol, as depicted in the two adjoining pictures. (D) Fura-2 intracellular calcium measurements of a single PC12 cell in response to 1 μM STS over the 480-min time course. A large spike of calcium is observed at a time corresponding to the coordinate release of YFP-cytochrome c into the cytosol, as depicted in the two adjoining pictures.

ilar increase in intracellular calcium, as in HeLa cells, but with a much longer timescale (11.5 h) and less prominent oscillations (data not shown). Similarly to HeLa cells, imaging of individual PC12 cells revealed calcium spikes immediately preceding the coordinate cytochrome c release (Figure 24-7D). Importantly, buffering of cytosolic calcium with BAPTA-AM in both HeLa and PC12 cells markedly reduced the release of cytochrome c from mitochondria (see Supplementary Information, Figure 24-S4), providing fur-

ther evidence that increases in cytosolic calcium coordinate the release of cytochrome *c* from mitochondria.

To demonstrate that the interaction between cytochrome *c* and InsP₃R is important *in vivo* for calcium release and apoptosis, we tested whether exogenously expressed cytochrome *c*-binding domain of InsP₃R (amino acids 2589–2749) would function as a dominant-negative inhibitor of STS-induced calcium oscillations and cytochrome *c* release in HeLa cells. As a negative control, we also examined the effects of exogenously expressed amino acids 923–1581 of the InsP₃R. We verified that the InsP₃R peptide fragments were expressed by Western blotting (Figure 24–8A). HeLa cells overexpressing either of the peptides exhibited no change in calcium release in response to 10 μ M ATP when compared with control (Figure 24–8B), demonstrating that endogenous InsP₃R function is not affected. YFP–cytochrome *c* HeLa cells were transfected with each peptide before testing for changes in calcium responses and cytochrome *c* release in response to 1 μ M STS (Figure 24–8C). Transfected cells were visualized by cotransfecting nuclear-targeted DsRed2. Expression of the C-terminal InsP₃R peptide (amino acids 2589–2749) markedly reduced the frequency and magnitude of the calcium transients induced by STS. Calcium transients were not observed for ~90 min in cells overexpressing the *c* terminus, whereas calcium transients began in control cells within minutes of STS exposure. Significant cytochrome *c* release was observed as early as 270 min in cells overexpressing the control peptide of the InsP₃R. Cytochrome *c* release was not detected in cells overexpressing the C-terminal peptide earlier than 12 h (data not shown), demonstrating that the C-terminal peptide markedly influences the kinetics of cytochrome *c* release in HeLa cells in response to STS treatment. The observed effects of the dominant-negative peptide may result from blocking cytochrome *c*-mediated activation of APAF-1. However, the peptide did not block cytochrome *c*-mediated caspase activation in cytosolic extracts (see Supplementary Information, Figure 24–S2A). Furthermore, broad-spectrum caspase inhibitors did not block translocation of cytochrome *c* to the InsP₃R (Figure 24–4A) or STS-induced calcium oscillations (see Supplementary Information, Figure 24–S2B). These results demonstrate that cytochrome *c* translocation to the endoplasmic reticulum and altered calcium homeostasis resulting in cell-wide cytochrome *c* release occur upstream of cytochrome *c*-mediated caspase activation.

Discussion

A major finding of our study is that cytochrome *c* binding to InsP₃R prevents calcium-dependent inhibition of InsP₃R function. The ability of low-micromolar concentrations of calcium to inhibit InsP₃R activity has been well-

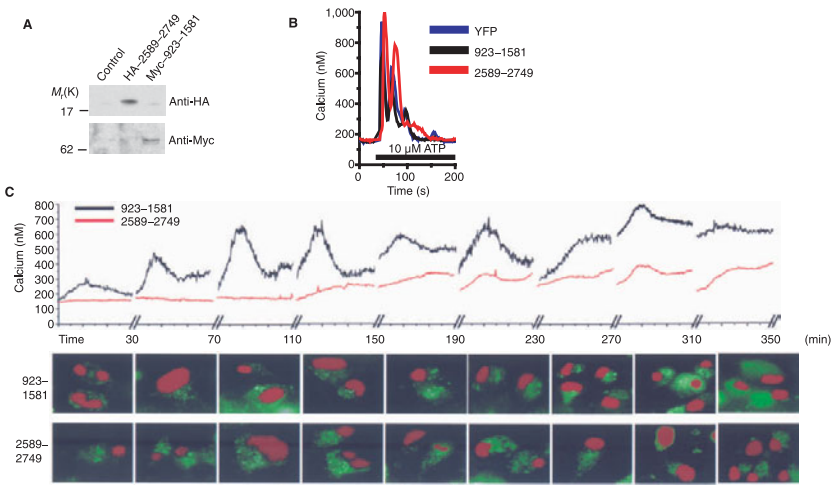


FIGURE 24-8. Cytochrome *c* binding to InsP_3R is required for STS-induced calcium oscillations and coordinate release of cytochrome *c*.

(A) Western blots demonstrating expression of Myc- InsP_3R -923–1581 and HA- InsP_3R -2589–2749 in HeLa cells. (B) Fura-2 intracellular calcium measurements in response to 10 μM ATP to stimulate the purinergic receptor in YFP-cytochrome *c* HeLa cells expressing either YFP alone (blue), YFP and Myc- InsP_3R -923–1581 (black), or YFP and HA- InsP_3R -2589–2749 (red). (C) Fura-2 intracellular calcium measurements in response to 1 μM STS in HeLa cells stably expressing YFP-cytochrome *c*, followed by cotransfection with InsP_3R peptides 923–1581 or 2589–2749. Transfection was monitored by nuclear-targeted DsRed. Marked oscillations are observed as early as 10 min in cells expressing peptide 923–1581, and continue until approximately 270 min. Oscillations are not observed in cells expressing peptide 2589–2749 for more than 150 min, and are reduced in magnitude. Cells displayed beneath each 30-min time course are representative of the distribution of YFP-cytochrome *c* (green) and nuclear DsRed (red).

characterized in multiple systems, including intact cells, and is thought to reflect a feedback mechanism to prevent excess release of calcium (9, 10). At a concentration of 1 nM, cytochrome *c* abolishes this action, an extremely high potency that supports physiologic relevance. By preventing calcium feedback inhibition of InsP_3R activity, cytochrome *c* would substantially amplify InsP_3R -mediated calcium release.

Our data support the hypothesis that altered calcium release by InsP_3R during apoptosis reflects direct binding of cytochrome *c* to the InsP_3R channel. Fluorescent imaging studies reveal that calcium spiking and cytochrome *c* release are temporally linked. Peptide competition of cytochrome *c* binding to

the InsP₃R demonstrates that the *in vivo* coordinate release of cytochrome *c* and altered calcium homeostasis are dependent on cytochrome *c* binding to the InsP₃R and initiating calcium release.

The coordinate release of cytochrome *c* from mitochondria throughout the cell has not been previously explained at the molecular level. We suggest that a feed-forward cycle of calcium release from InsP₃R and cytochrome *c* release from mitochondria provides a molecular mechanism. Cytochrome *c* binding to InsP₃R adjacent to mitochondria would sensitize InsP₃R to increased calcium release, causing mitochondrial and cytosolic calcium overload and further cytochrome *c* release. Therefore, small amounts of cytochrome *c* released early in apoptosis would first function at endoplasmic reticulum membranes to alter calcium handling. Subsequent global cytochrome *c* release from all mitochondria would supply the cytosolic cytochrome *c* necessary for caspase activation and completion of the apoptotic cascade.

Our findings reconcile previous reports suggesting a gradual apoptotic release of cytochrome *c* into the cytosol (21, 22) with reports suggesting coordinate release (6). The presumed cytosolic fractions in some earlier studies (21, 22) were 10,000g supernatants, which contain endoplasmic reticulum. Here we show this fraction to be the principal target of early cytochrome *c* release. Imaging studies have revealed rapid and coordinate release of cytochrome *c* into the cytoplasm (6). As cytochrome *c* binds to InsP₃R in close apposition to mitochondria, cytochrome *c* released and bound to endoplasmic reticulum membranes would not be resolved by confocal or epifluorescence microscopy. We hypothesize that a slow, graded release of cytochrome *c* primes InsP₃R for increased calcium release, eventually triggering the massive cell-wide cytochrome *c* release coordinated through a calcium transient.

The importance of InsP₃R in apoptosis has been emphasized by numerous studies using genetic manipulations to reduce InsP₃R levels. Type-1-InsP₃R-deficient lymphocytes are resistant to apoptotic stimuli (23), and treatment of cells with type-3 InsP₃R antisense RNA retards apoptosis (24, 25). DT40 B cells lacking all forms of InsP₃R display reduced apoptotic cell death (16). Although these cells are resistant to apoptosis, almost a third do progress through the programmed cell death pathway, perhaps through increased cytosolic cytochrome *c* activating APAF-1 in the absence of increased cytosolic calcium. Alternatively, calcium release through InsP₃R may function as an apoptotic signal amplifier, as suggested for cytochrome *c* (26, 27). If true, apoptosis could still occur in the absence of InsP₃R, albeit much less efficiently.

Methods

Materials. STS, MDL-28170, and Z-VAD-FMK were purchased from BIO-MOL Research Laboratories, Inc. (Plymouth Meeting, PA). Complete protease inhibitor cocktail without EGTA was from Roche Applied Science (Indianapolis, IN). Cytochrome *c*-agarose, BSA-agarose, protein A-Sepharose, and all other chemicals were from Sigma (St. Louis, MO).

Yeast Two-Hybrid. The Matchmaker 3 yeast two-hybrid system from Clontech (Palo Alto, CA) was used. AH109 β -gal yeast was used. InsP₃R fragments were cloned into pGADT7 (β -gal acceptor domain) vector and were screened against a rat brain and human fetal kidney library (Clontech) in accordance with the manufacturer's specifications. Expression of these fragments was determined by Western blotting using antibodies from Clontech, corresponding to the expression vector. Positive clones grew on minimal SD agar (Clontech) lacking adenine, histidine, leucine, and tryptophan, and had β -gal activity.

GST Pulldown Assay. GST-InsP₃R proteins (5 μ g) were incubated with 5 μ g cytochrome *c* in 500 μ L binding buffer (150 mM NaCl, 50 mM Tris at pH 7.8, 1% Triton X-100 and 1 mM EDTA) for 1 h at 4°C with rotation. 50 μ L of 50% GST-Sepharose (Amersham Biosciences, Piscataway, NJ) was added and the incubation was continued for 30 min at 4°C. The beads were washed four times with 1 mL binding buffer and separated on an SDS-PAGE gel. One half of the gel containing the GST fusion proteins was stained with Coomassie Blue, and the remainder of the gel containing cytochrome *c* was transferred to nitrocellulose and blotted.

Calcium Release Measurements. Calcium release through recombinant type-I (SII+) InsP₃R was measured exactly as described previously (11). Calcium concentrations in all solutions were calibrated with a calcium-selective mini-electrode, and confirmed with fluorescent dyes. When the effects of cytochrome *c* were examined, it was added simultaneously with InsP₃. In the absence of InsP₃, SERCA activity was not affected by the addition of cytochrome *c*. At the concentrations used in this study, cytochrome *c* did not change the calcium concentration of the assay solution, as determined with a calcium mini-electrode.

Cell Lines. Human HeLa cells were obtained from the ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin. PC12 cells were cultured in DMEM supplemented with 10% horse serum, 5% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. DT40-WT and DT40-KO cells (16) were cultured in RPMI 1640 supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, 0.5 mg mL⁻¹ G418, 1 mM glutamine, and 1% penicillin-streptomycin. PC12 and HeLa cells stably expressing YFP-cytochrome *c* were generated by transfection with *Pvu*I-linearized plasmid and selection in G418, exactly as suggested by the plasmid manufacturer (pcDNA3.1; Invitrogen). HeLa and

PC12 cells were selected and maintained in 1 mg mL^{-1} and 1.5 mg mL^{-1} G418, respectively. YFP-cytochrome *c* expression was confirmed in isolated clones by confocal microscopy and Western blotting. Mitochondrial localization was confirmed by colocalization with MitoTracker Red (Molecular Probes, Eugene OR).

Expression Constructs. *InsP₃R* and *SERCA-2b*: The type-I *InsP₃R* SII+ splice variant and *SERCA-2b* in pcDNA3.1 have been described elsewhere (11) and were kindly provided by S.K. Joseph (Thomas Jefferson University, Philadelphia, PA).

CFP-InsP₃R: Type-I *InsP₃R* was digested with *Xba*I and sequentially partially digested with *Kpn*I through use of an internal *Kpn*I site in the *InsP₃R* sequence. The ~9,300 bp insert was then subcloned into the *Kpn*I-*Xba*I sites of pECFP-C1 (Clontech). The pECFP-C1 plasmid was propagated in the *dam*-*E. Coli* strain SCS110 (Stratagene, La Jolla, CA) because of overlapping Dam methylation of the *Xba*I site. This construct expresses CFP as an N-terminal fusion with the *InsP₃R*. This fusion was chosen because its N terminus is physically associated with the C-terminal tail of the *InsP₃R* (28). This fusion protein was localized to endoplasmic-reticulum-like membranes, as determined by confocal microscopy (data not shown).

YFP-cytochrome c: EYFP was fused to the N terminus of cytochrome *c* using single-overlap extension (SOE). This required PCR of EYFP and cytochrome *c* separately, then combining the two templates into a single PCR reaction using a 5' primer at the beginning of the EYFP sequence and a 3' primer on the cytochrome *c* sequence. The overlap region was engineered onto the reverse EYFP primer and forward cytochrome *c* primer, and contains *Bgl*II, *Xho*I, and *Hind*III restriction sites necessary for facilitating removal and insertion of other cDNAs. PCR primers for EYFP were 5'-AATGCTAGCGCCACCATGGTGAGCAAGGGCGAG-3' (EYFP-F) and 5'-CGAAGCTTGAGCTCGAGATCTGAGCTTGACAGCTCGTCCAT-3' (EYFP-R). PCR primers for cytochrome *c* were 5'-CTCAGATCTCGAGCTCAAGCTTCGATGGGTGATGTTGAAAAAG-3' (CytC-F) and 5'-AATTCTAGATTATTATTAGTAGCCTTTT-3' (CytC-R). EYFP-F contains an *Nhe*I site and a Kozak sequence, and CytC-R contains a stop codon and *Xba*I site. The cDNA for YFP was from Clontech, and cytochrome *c* was isolated from a rat brain cDNA library (Clontech) using primers CytC-F and CytC-R, and was sequenced to confirm that no mutations were generated by PCR. The final PCR product was subcloned into the *Nhe*I and *Xba*I sites of pcDNA3.1 (Invitrogen).

C-terminal-tail: Amino acids 2589–2749 of the rat type-I *InsP₃R* were amplified using PCR, incorporating a 5' *Eco*R1 and 3' *Not*I restriction sites and cloned in frame to the *Eco*R1-*Not*I sites of pCMV-HA (Clontech). PCR primers used were 5'-TTAAGAATTCCGATCGACACCTTTGCTGACCTG-3' and 5'-TTTTGCGGCCGCTTAGGCTGGCTGCTGTGGGT-3'.

InsP₃R-1 923–1581: Amino acids 923 to 1581 of the rat type I *InsP₃R* were amplified using PCR incorporating a 5' *Eco*R1 and 3' *Xho*I restriction sites and cloned in-frame to the *Eco*R1-*Xho*I sites of pCMV-Myc (Clontech). PCR primers used were 5'-GCA GAA TTC GGT CTA TCC ATG GAG TTG GG-3' and 5'-GCA CTC GAG GCG GGC TGA TAA CCG CCA GTT-3'.

Antibodies. Rabbit polyclonal antiserum against the type-I InsP_3R was generated by injecting New Zealand White male rabbits with the peptide CRIGLLGHPPHMNVNPQQPA coupled to keyhole limpet hemocyanin (Pierce Biotechnology, Rockford, IL). Initial injection was with Complete Freund's Adjuvant, and boost injections at days 14, 21, and 49 were with Incomplete Freund's Adjuvant. Rabbit injections, bleeds, and housing were performed by Cocalico Biologicals (Reamstown, PA). Antisera generated against this peptide have been reported previously (29) and are specific for the type-I InsP_3R . The production of rabbit anti-HO2 antisera has been described previously (30). Mouse anti-Chicken IgM clone M-4 was from Southern Biotech (Birmingham, AL). Mouse anti-cytochrome *c* was purchased from Zymed Laboratories (San Francisco, CA). Mouse anti-cytochrome *c* oxidase subunit IV (CytOx) was from Molecular Probes. Rabbit anti-Prohibitin was from Research Diagnostics (Flanders, NJ). Goat anti-lactate dehydrogenase (LDH) was purchased from Chemicon (Temecula, CA). Mouse anti-PARP was from Alexis Biochemicals (San Diego, CA). Mouse anti-sarco-endoplasmic reticulum ATPase-2 (SERCA2) was from Affinity BioReagents (Golden, CO).

Subcellular Fractionation. After inducing apoptosis with STS or anti-IgM treatment, cells were harvested by gently scraping plates with a cell scraper and washing once with cold PBS. The washed pellet was subjected to one freeze-thaw cycle in liquid nitrogen. Pellets were resuspended in 1 mL buffer A (250 mM sucrose, 10 mM Tris-HCl at pH 7.5, 1 mM EGTA, 1 mM phenyl methylsulphonyl fluoride [PMSF], and one protease inhibitor pill). Cells were then homogenized on ice using a 1-mL glass Dounce homogenizer with a tight-fitting pestle until ~95% of cells were disrupted, as judged by Trypan blue staining. Crude lysates were centrifuged at 1,000g for 15 min at 4°C to remove nuclei and unbroken cells. The supernatant was collected and the pellet (P1) discarded. The low-speed supernatant was then subjected to a 10,000g centrifugation for 15 min, yielding the 10,000g pellet (P2). The supernatant from the P2 pellet was centrifuged at 15,000g to completely rid the supernatant of any remaining mitochondria. Finally, the 15,000g supernatant was separated into cytosol (S3) and light membrane (P3) fractions by centrifugation at 100,000g for 1 h. The 100,000g supernatant was collected as the S3 fraction and the pellet was resuspended in 40–70 μL of buffer A. The P2 fraction was washed twice by resuspending cells in 100 μL buffer A and pelleting (10,000g for 15 min). After the final wash, the P2 fraction was resuspended in 50–100 μL buffer A. All fractionations were repeated a minimum of four times with essentially identical results.

Co-immunoprecipitation. Cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.8, 1% Triton X-100, and 1 mM EDTA) was added to 100 μg of P3 fraction to bring samples to a total volume of 500 μL . Samples were cleared of insoluble debris by centrifugation at 10,000g. Anti- InsP_3R -1, pre-immune sera, or anti-SERCA2 antibodies and protein A-Sepharose beads were added and incubated on a rotator for 30 min at 4°C. The protein A-Sepharose beads were washed three times with lysis buffer and quenched with 20 μL of SDS sample buffer. Co-immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting.

Cytochrome *c*-Agarose Binding. Cerebellar microsome or COS-7 cell lysates were incubated with 20 μ L cytochrome *c*-agarose (approximately 200 μ g total cytochrome *c*) in a final volume of 0.5 mL for 2 h at 4°C in the dark with rotation. Beads were washed four times with 0.5 mL cell lysis buffer and separated on SDS-PAGE gels. BSA coupled to the same agarose matrix as cytochrome *c* was used as a negative control. When used, InsP₃Rs were purified to homogeneity exactly as described previously (31).

Caspase Activity. Caspase activity was performed on S3 fractions using the EnzChek caspase-3 assay kit #1, essentially after the manufacturer's protocol (Molecular Probes). This kit measures the increase in fluorescence generated by the cleavage of the aminomethylcoumarin (AMC)-labeled caspase-3 substrate Z-DEVD-AMC. The production of fluorescent substrate was monitored continuously every 5 min for approximately 2 h in a 96-well dish using an automated fluorescent plate reader with data acquisition software (LS55 luminescence spectrometer, PerkinElmer Instruments, Shelton, CT). The slope of the linear regression drawn through each time point was used to determine change in fluorescence over time for each sample. A standard curve using known amounts of AMC was used to convert fluorescent values to specific catalytic activity.

FRET. PC12 cells stably expressing YFP-cytochrome *c* were cultivated in glass bottom 35-mm dishes (MatTek Corporation, Ashland, MA). CFP-InsP₃R and ECFP cDNAs were transfected with Lipofectamine 2000 (Invitrogen), and were used in experiments 48 h after transfection. Cells were imaged on a Zeiss Axiovert 135 inverted microscope with a 40 \times Plan-Neofluar lens (Carl Zeiss MicroImaging, Thornwood, NY) equipped with a Photometrics Coolsnap HQ cooled charge-coupled device camera (Roper Scientific, Tucson, AZ). Cells were imaged in phenol-free Leibovitz's L-15 medium supplemented with 10% horse serum, 5% fetal bovine serum, and 2 mM L-glutamine overlaid with mineral oil at 37°C with a heated stage. Dual emission ratiometric imaging was controlled with IPLab 3.5.5 software (Scanalytics, Inc., Fairfax, VA) using excitation filter D436/10, beam splitter 86002bs, and emission filters D470/30 and HQ535/30 (Chroma Technology Corp., Brattleboro, VT). Emission filters were alternated using a computer-controlled filter wheel (LUDL Electronics, Hawthorne, NY). Cells were excited at 436 nm and 535:470-nm ratiometric images were acquired every 5 min for 350 min. Data in Figure 24-6C and D were pooled from at least three separate experiments for each trace (*n* values are indicated).

Calcium Imaging. Calcium measurements were performed as previously described (32) using the Intracellular Imaging calcium imaging system and InCyt2 imaging software (Intracellular Imaging, Cincinnati, OH). Fura-2 AM-loading (Molecular Probes) was performed for 1 h at 37°C for PC12 cells stably expressing YFP-cytochrome *c*, and 15 min at 37°C for HeLa cells stably expressing YFP-cytochrome *c*. Cells were treated with 1 μ M STS at time zero and incubated at 37°C until imaging began. Resting calcium levels in cell lines were similar: that is, 100–200 nM. Images at 340 nm, 380 nm, and 485 nm were collected every 10 s for 30 min in HeLa cell experiments and

every 20 s for 1 h in PC12 cell experiments. A 10-min gap between each time point was necessary for data acquisition and compilation. All measurements shown are an average of 50 cells, unless otherwise stated, and are representative of a minimum of three, and in most cases, a larger number of independent experiments.

ERRATUM: In Boehning et al (Nat Cell Biol 5:1051–1061, 2003), the units for panels (A) and (B) in Figure 24–5, and the legend referring to these panels, should have read $\mu\text{g mL}^{-1}$. [*The correction has been made in this reprinted version.*—Editor.]

NOTE: The Supplementary Information at the end of this chapter appears on the *Nature Cell Biology* Web site (<http://www.nature.com/naturecellbiology>).

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Supplementary Information

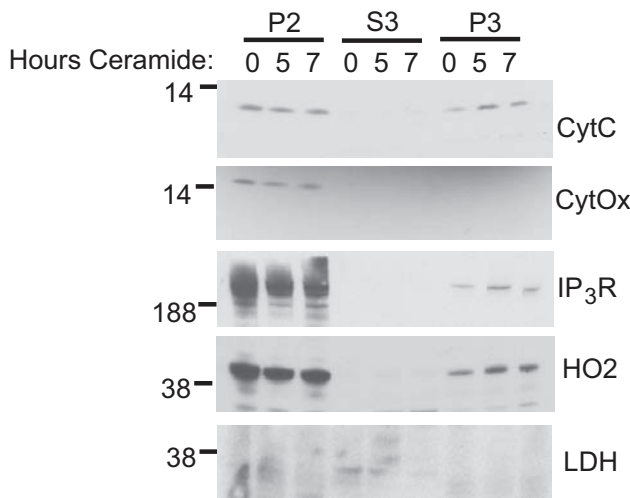


FIGURE 24-S1. Cytochrome *c* translocates to the ER binding IP₃R during ceramide induced apoptosis in HeLa cells.

Cytochrome *c* translocates from the mitochondria (P2) to the ER (P3) fraction during apoptosis. Apoptosis was induced with 100 μ M C2-ceramide for 0, 5, or 7 h in serum free medium. Cells were harvested, fractionated, and processed as described in Materials and Methods. Fraction loading controls cytochrome *c* oxidase (CytOx, mitochondria), heme oxygenase-2 (HO2, ER), and lactate dehydrogenase (LDH, cytosol) were used to check purity of the fractions and to ensure equal protein distribution in all lanes.

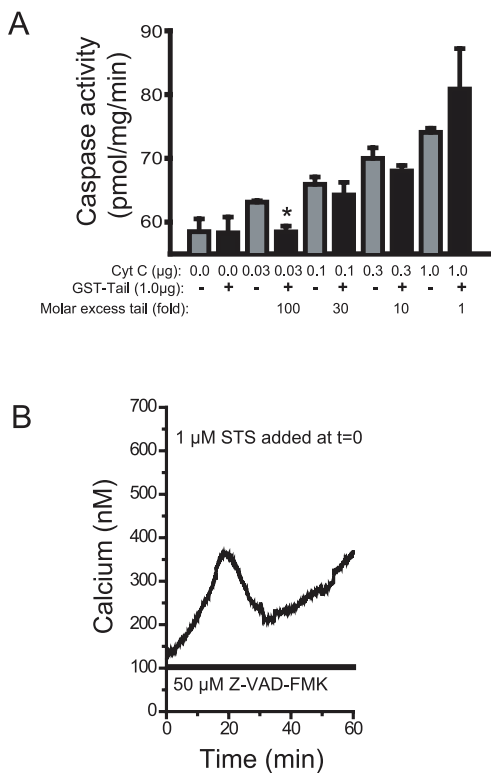


FIGURE 24-S2. Effects of cytochrome c on Ca²⁺ homeostasis are independent of caspase activation.

(A) Caspase inhibition does not block Ca²⁺ oscillations induced by STS. Fura-2 intracellular Ca²⁺ measurements in response to 1 μM STS in HeLa cells stably expressing YFP-cytochrome c. Cells were pretreated with 50 μM Z-VAD-FMK for 10 min before the addition of 1 μM STS. Ca²⁺ oscillations and frequency were unaffected by caspase blockade. (B) The dominant-negative peptide does not prevent cytochrome c-mediated activation of the apoptosome in cytosolic extracts. HeLa cells were homogenized with a Dounce homogenizer for 80 strokes in homogenization buffer (250 mM sucrose, 20 mM HEPES 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail). Homogenates were centrifuged at 10,000g for 5 min, and the supernatant centrifuged at 100,000g for 30 min. The supernatant (35 μg) supplemented with 1 mM dATP was used for subsequent caspase assays. Caspase assays on cytosolic extracts were performed as described in Materials and Methods with varying amounts of cytochrome ± GST-IP₃R 2589–2749. Significant inhibition of caspase-3 activation by cytochrome c was only observed when a 100-fold molar excess of GST-IP₃R 2589–2749 was used (indicated by an asterisk; P<0.05).

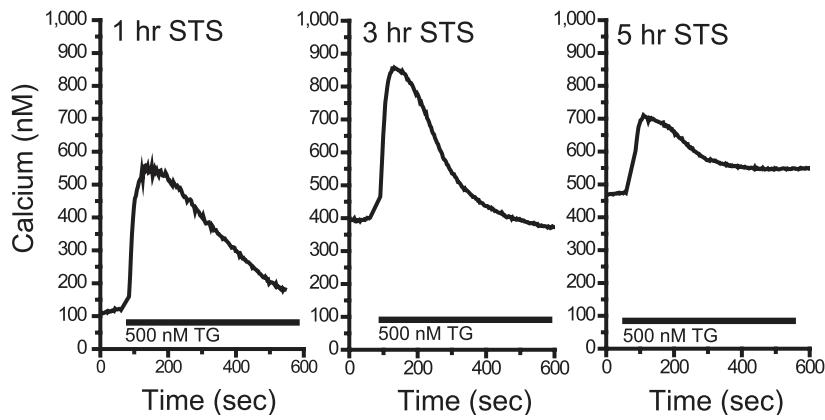


FIGURE 24-S3. Staurosporine treatment does not significantly compromise the integrity of ER Ca²⁺ stores.

Fura-2 intracellular Ca²⁺ measurements in response to 1 μ M STS in HeLa cells stably expressing YFP-cytochrome *c*. Cells were pretreated with STS for 1, 3, and 5 hours followed by 500 nM thapsigargin to block SERCA activity.

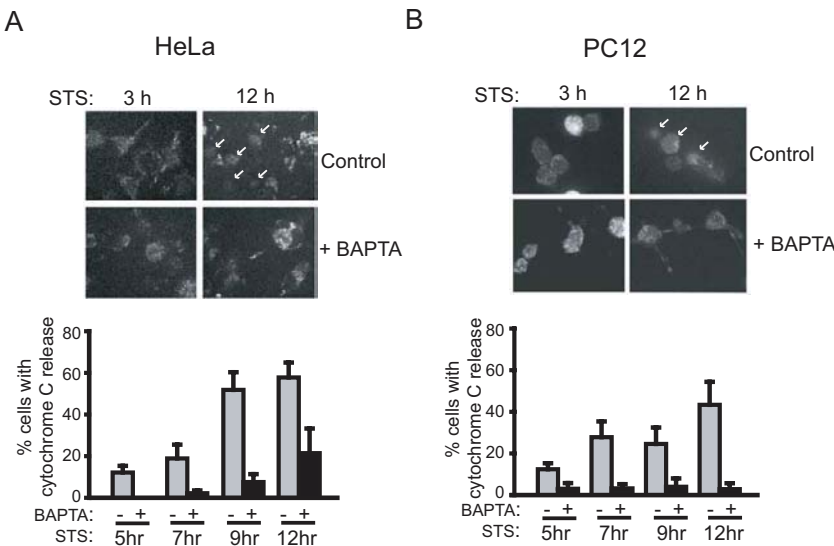


FIGURE 24-54. Cytosolic Ca^{2+} buffering inhibits release of cytochrome *c* in response to STS treatment.

HeLa cells stably expressing YFP-cytochrome *c* HeLa were loaded with 2 μM BAPTA-AM for 30 min at room temperature. Cells were then treated with 1 μM STS and monitored for cytochrome *c* release by confocal microscopy. Top panels are representative images of cells 3 h and 12 h after STS treatment. Cells with cytochrome *c* release are indicated by a white arrow. At each time point, at least 120 cells from 5 different fields were analyzed for cytochrome *c* release and the total number of cells pooled. Time-course and statistical comparison of cytochrome *c* release in HeLa cells \pm BAPTA-AM treatment are represented below by the bar graph. PC12 cells stably expressing YFP-cytochrome *c* were loaded with 2 μM BAPTA-AM, treated with STS, and then monitored for cytochrome *c* release as in Panel (A).

CHAPTER 25

Bilirubin Benefits

Cellular Protection by a Biliverdin Reductase Antioxidant Cycle

Thomas W. Sedlak
Solomon H. Snyder

Bilirubin is widely known as an end product of heme metabolism. Very high levels of serum bilirubin lead to its accumulation in the brain, causing kernicterus (1, 2). Almost all newborns display some level of jaundice, and some display high enough serum bilirubin levels that phototherapy or exchange transfusion is considered.

What most of the medical profession has not appreciated is that, from a teleologic perspective, biosynthesis of bilirubin as the key catabolite of heme does not seem to make sense. Bilirubin is a secondary degradation product of heme. Heme is best known as a constituent of hemoglobin, which is released in association with the breakdown of aging red blood cells. Heme also is contained in a wide range of enzymes whose turnover also leads to free heme release. Free heme can be toxic, so nature evolved a family of heme oxygenase enzymes to degrade heme (3, 4), and their blockade leads to greatly increased excretion of unmetabolized heme in the bile (5). These enzymes cleave the heme ring to form biliverdin, iron, and a 1-carbon fragment as car-

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bon monoxide (CO) (Figure 25–1). CO is increasingly appreciated as a neurotransmitter (6, 7) and iron, itself toxic, is excreted from cells by a recently characterized pump (8, 9, 10, 11). Biliverdin would seem to be an appropriate end product of the pathway, being readily excreted in the bile to enter the intestine and leave the body in the feces. Indeed, in birds, reptiles, and amphibians, biliverdin is the predominant end product of heme degradation (12). For reasons that until now have seemed obscure, in mammals, biliverdin undergoes additional metabolism, being reduced by biliverdin reductase (BVR) to bilirubin, a step that consumes the energy resource nicotinamide adenine dinucleotide phosphate (NADPH) (13). As bilirubin is more hydrophobic and insoluble than biliverdin, it is glucuronidated to facilitate excretion into the bile, costing additional cell resources.

Why have mammals evolved an energetically expensive and apparently unnecessary enzymatic step to converting the relatively innocuous biliverdin to the more toxic bilirubin? Moreover, why would nature develop a system that generates “elevated” bilirubin levels in a high proportion of all neonates? Nature may not be altogether foolhardy, as the mildly to moderately elevated levels of bilirubin in neonates are not always toxic. In 1965, in this journal, Wishingrad and associates (14, 15) argued that hyperbilirubinemia of premature infants is not as deleterious as previously thought. Furthermore, some individuals with the impaired bilirubin glucuronidation system of type 2 Crigler-Najjar syndrome maintain bilirubin levels of 19 mg/dL for 50 years without detectable damage to the nervous system (16).

Some authors have suggested that unconjugated bilirubin is physiologically useful, because it can cross the placenta, moving from the fetal to the maternal circulation easier than biliverdin (17, 18, 19). However, isomer specificities of fetal and maternal BVR differ. The principal isomer of early fetal bilirubin is IX β , whereas the adult forms bilirubin IX α (20, 21). Hence, adult BVR cannot have evolved to service needs of the fetus.

One possible physiologic role for bilirubin is as an antioxidant. As early as the 1950s, bilirubin was reported to protect against the oxidation of lipids such as linoleic acid and vitamin A (22, 23, 24). In the late 1980s, Ames and colleagues (25, 26) demonstrated that the antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation. Serum concentrations of bilirubin are high enough to account for a substantial portion of the total antioxidant capacity of serum (27, 28). Thus, bilirubin might alleviate oxidant stress in the blood. However, what matters most is what goes on inside cells. During the oxidant stress associated with myocardial and cerebral infarcts, infection, inflammation, and various causes of ischemia, the intracellular environment is exposed to high concentrations of reactive oxygen species. It has long been assumed that the principal cellular antioxidant is the peptide glutathione (GSH), whose tissue concentrations are millimolar, presumably

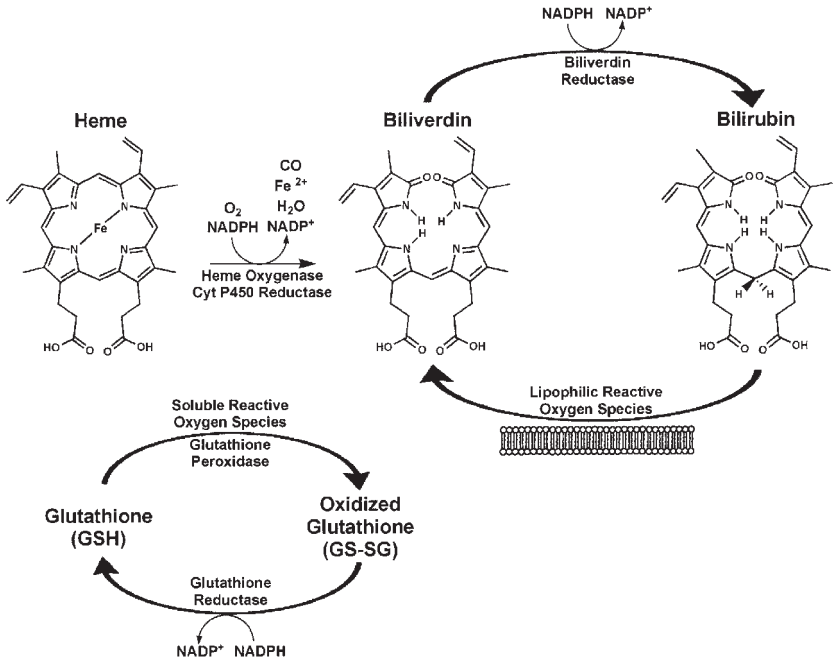


FIGURE 25-1. Oxidation-reduction cycles for bilirubin and GSH.

Lipophilic reactive oxygen species act directly on bilirubin, leading to its oxidation to biliverdin. BVR catalyzes the reversion of biliverdin to bilirubin, permitting bilirubin to detoxify a 10,000-fold excess of oxidants. Soluble oxidants are detoxified by GSH, a cycle that requires 2 enzymes, GSH peroxidase and GSH reductase.

sufficient to cope with most instances of oxidative stress. By contrast, levels of bilirubin in rodent tissues are only 10 to 50 nanomolar, at least 10,000 times lower than concentrations of GSH (D. Baranano and S.H. Snyder, unpublished observations).

Biliverdin Reductase Cycle

Insight into a mechanism whereby low nanomolar concentrations of bilirubin can protect cells came from studies of the heme oxygenase system in the brain. Heme oxygenase was first characterized by Schmid and associates in the 1960s (29). Maines and associates (30, 31) purified and cloned a family of heme oxygenase isozymes. The first known form of heme oxygenase, HO1, is highly concentrated in the spleen, the repository of aging red blood cells. HO1 is a remarkably inducible enzyme, with its synthesis rapidly and profoundly stimulated by more stimuli than almost any other known inducible enzyme (32). Heme itself, released from degrading red blood cells, is a

major stimulus to HO1, accounting for the rapid removal of free heme from the circulatory system. A second form of the enzyme, HO2, is not inducible and is highly concentrated in neurons in discrete regions of the brain, where it forms CO as a neurotransmitter (3, 33).

A role for HO2 in protecting neurons from oxidative damage comes from studies using mice with genetic deletion of HO2. Such mice are much more susceptible to stroke damage elicited by ligation of the middle cerebral artery, whereas mice with knockout of HO1, which are far more debilitated than the HO2 knockouts, do not manifest increased stroke damage (34, 35). Similarly, HO2-deficient animals demonstrate greater brain tissue loss and diminished motor function after traumatic brain injury (36). Brain cultures of HO2 knockout mice are also exceptionally susceptible to apoptotic death elicited by oxidative stresses such as hydrogen peroxide. Remarkably, as little as 10 nanomolar bilirubin can protect cultures from the oxidant stress of 10,000 times higher concentrations of hydrogen peroxide (37).

How might one explain this paradox? One possibility would involve cycling between biliverdin and bilirubin. According to this hypothesis, when a molecule of bilirubin acts as an antioxidant, it is itself oxidized to biliverdin. BVR is an abundant and ubiquitous enzyme with a high turnover rate. Hence, endogenous BVR should suffice to reduce newly formed biliverdin back to bilirubin. The intrinsic amplification properties of enzymes could readily augment the antioxidant effects of bilirubin 10,000-fold. Such a cycle would represent an elegant tour de force on the part of nature, making use of bilirubin's antioxidant capacity but ensuring that tissues had low endogenous levels of bilirubin, as the micromolar levels necessary for direct antioxidant actions would be toxic.

To establish the function of this cycle, we first showed that the isomer of biliverdin generated by reactive oxygen species is the IX α form, which can serve as a substrate for BVR (38). We then directly examined whether BVR is required by cells to protect against reactive oxygen species. Using the technique of RNA interference, we depleted cells of BVR, leading to a tripling of reactive oxygen species levels and a marked augmentation of cell death. We compared the roles of cellular bilirubin and GSH, depleting the latter with buthionine sulfoximine, an inhibitor of the GSH-synthesizing enzyme γ -glutamylcysteine synthase. Lowering GSH levels 95% led to only a 50% increase in reactive oxygen species and a much more modest increase in cell death.

Might bilirubin and GSH play distinct but complementary roles in protecting cells? Being lipid soluble, bilirubin might primarily protect cells against lipid peroxidation. By contrast, the water-soluble GSH might protect soluble proteins from oxidation.

Besides bilirubin and GSH, cells seem to use several other systems to protect against oxidative stress. Examples include the enzymes superoxide dis-

mutase and catalase, which act in concert to convert superoxide to water. Direct antioxidant actions are elicited by ascorbate and vitamin E.

Clinical Relevance

A variety of evidence is accumulating that mild to moderately elevated serum bilirubin levels are associated with better outcome in diseases involving oxidative stress (Table 25–1). Neonates have long been the principal concern in studies of serum bilirubin levels. Neonatal Gunn rats, which have elevated unconjugated bilirubin as a result of autosomal recessive lack of glucuronyl transferase, are resistant to oxidative damage when reared in hyperoxia (39). Premature neonates who are treated with supplemental oxygen often manifest retinal damage, presumably associated with oxidative stress. Heyman et al. (40) and Yeo et al. (41) observed a diminished incidence of retinopathy of prematurity in infants with elevated serum bilirubin, although some studies fail to detect such relationships (42–47).

Insight into the mechanism of bilirubin protection comes in studies that have monitored the rate of rise in serum bilirubin in the first few days of life in infants with illnesses that are associated with free radical production, such as circulatory failure, neonatal asphyxia, aspiration, and sepsis (48, 49). The rate of bilirubin rise was less in patients than in a control group, suggesting that bilirubin is consumed to cope with oxidative stress. Others have found that bilirubin levels correlate with total antioxidant capacity in blood of neonates (27, 28, 50, 51) and children with sickle cell disease (52), although this association was not found in one study (53).

In adults who trained extensively to participate in 50- and 80-kilometer marches, bilirubin and uric acid levels were increased as was resistance to protein oxidation (54). Patients with long-standing amyotrophic lateral sclerosis have lower bilirubin levels than patients with more recently diagnosed disease (55). This finding fits with abundant evidence that the pathophysiology of amyotrophic lateral sclerosis involves oxidative stressors that may consume bilirubin. Oxidative metabolites of bilirubin are increased in the urine of patients with sepsis (56) and exacerbations of atopic dermatitis (57).

Several workers have examined a link between serum bilirubin and coronary artery disease. Two studies involving the offspring and spouses of the original Framingham cohort evaluated the relationship of bilirubin and the risk of myocardial infarction in >5,000 participants (58, 59). Higher bilirubin levels, ~15.4 μM (0.9 mg/dL), were associated with lowered risk of myocardial infarction and other cardiovascular disease events compared with individuals with lower bilirubin levels of ~8.6 μM (0.5 mg/dL). In a case-control study, Hopkins et al. (60) compared familial coronary artery disease patients with control subjects. The diseased individuals displayed substan-

TABLE 25-1. Summary of studies that examined the relationship of bilirubin to human disease

| Clinical condition | Statistically beneficial bilirubin effect | Study characteristics | Reference |
|---------------------------------|--|---|-----------|
| Antioxidant status: neonates | Yes | 44 infants with free radical-producing diseases (respiratory distress, circulatory failure, sepsis, aspiration, and asphyxia) had significantly lower rises in serum bilirubin than neonates who were ill from nonoxidative disease. | 48 |
| | Yes | 25 preterm infants with oxygen-radical diseases (intraventricular hemorrhage, retinopathy, bronchopulmonary dysplasia, and necrotizing enterocolitis) were found to have significantly lower levels of bilirubin than 57 control cases. | 49 |
| | Yes | Plasma bilirubin levels were closely correlated with antioxidant levels with bilirubin levels in term infants ($r^2=0.774$), although less so in preterm infants. | 28 |
| | Yes | In 8 newborns with hyperbilirubinemia (250–435 μM), exchange transfusion decreased both bilirubin levels and plasma antioxidant capacity. | 27 |
| | No | In 22 jaundiced preterm infants, bilirubin was not associated with plasma antioxidant status. Individuals with elevated liver function tests were not excluded. | 53 |
| | Yes | In 28 preterm infants, the antioxidants bilirubin (~ 71 – $111 \mu\text{M}$) and uric acid were correlated with total plasma antioxidant levels. | 50 |
| Antioxidant status: adults | Yes | Plasma from infant blood was oxidized less than that from adult blood, correlating with their respective bilirubin levels. | 51 |
| | Yes | After extensive training and physical exercise, 31 male subjects demonstrated increased levels of bilirubin and uric acid and decreased measures of oxidative damage in serum. | 54 |
| | Yes | Bilirubin oxidative metabolites were significantly elevated in the urine of 19 septic patients compared with 28 control subjects. | 56 |

TABLE 25-1. Summary of studies that examined the relationship of bilirubin to human disease *(continued)*

| Clinical condition | Statistically beneficial bilirubin effect | Study characteristics | Reference |
|----------------------------------|--|--|-----------|
| Amyotrophic lateral sclerosis | Yes | Patients with long-standing disease have lower bilirubin than more recently diagnosed individuals. | 55 |
| Atopic dermatitis | Yes | 13 children with exacerbations of atopic dermatitis had significantly elevated bilirubin oxidative metabolites in their urine, compared with 28 matched control subjects. | 57 |
| Cancer | Yes | In a 10-y follow-up of >10,000 Belgians, risk of death from cancer, especially nonlung cancer, decreased with increasing serum bilirubin. | 75 |
| Coronary artery disease | Yes | In 877 asymptomatic male Air Force pilots, lower bilirubin levels were correlated with greater risk and severity of coronary artery disease. | 61 |
| | Yes | A U-shaped relationship between bilirubin levels and risk of ischemic heart disease was found in 7,685 British men. The greatest risk for heart disease was found with low bilirubin levels. | 63 |
| | Yes | The relationship between bilirubin level and risk of familial coronary artery disease was studied in 314 men and women. Higher bilirubin levels were associated with protection, and cigarette smoking attenuated this effect. | 60 |
| | Yes | In a 3-y genetic study of 1,240 Utah adults, individuals with early-onset coronary artery disease had significantly lower bilirubin levels. | 69 |
| | Yes | An inverse relationship of bilirubin levels and risk of angiography-proven coronary artery disease was demonstrated in 254 male patients. | 62 |
| | Yes | Framingham Offspring Study: In a cohort of 5,124 individuals, bilirubin levels were inversely related to risk of myocardial infarction and overall cardiovascular disease. | 58, 59 |

TABLE 25-1. Summary of studies that examined the relationship of bilirubin to human disease *(continued)*

| Clinical condition | Statistically beneficial bilirubin effect | Study characteristics | Reference |
|--------------------------------|--|---|-----------|
| Peripheral vascular disease | Borderline | In 328 participants of the Family Heart Study, decreases in bilirubin of 1 μ M were associated with increased heart disease in male participants, although the finding was not statistically significant ($P=0.056$). | 70 |
| | Yes | In a 3-y study of 50 participants with hyperbilirubinemia of Gilbert's syndrome, prevalence of coronary artery disease was 2%, compared with 12.1% in a control population. | 66 |
| | Yes | In 31 individuals with peripheral vascular disease, bilirubin levels were significantly lower than normal population levels. | 72 |
| | Yes | In 1,741 subjects who underwent screening for carotid artery disease, bilirubin levels in the highest quartile were linked to a 32% reduction in risk of plaques. | 73 |
| Retinopathy of prematurity | Yes | Lower bilirubin concentrations were associated with worsened retinopathy of prematurity in 45 infants, even when gestational age was controlled for. | 40 |
| | No | Lower bilirubin concentrations were found in more severe cases of retinopathy of prematurity, but not when gestational age was controlled. | 42 |
| | No | Bilirubin levels were not related to retinopathy of prematurity in 151 neonates. | 43 |
| | No | In 24 infants, no protective effect of bilirubin was found for retinopathy of prematurity. | 44 |
| | Yes | In 128 premature infants, lower peak bilirubin levels were associated with greater vision loss. | 41 |
| | No | No relationship was found for bilirubin levels and retinopathy of prematurity in 157 infants of gestational age 23–26 weeks. | 46 |
| | No | Bilirubin levels were unrelated to retinopathy of prematurity in 76 infants. | 45 |
| | No | Elevated bilirubin levels did not protect and possibly predisposed to retinopathy of prematurity in 240 very low birth weight infants. | 47 |

tially lower serum bilirubin levels than the control subjects. The protective effect of bilirubin on coronary artery disease risk in this population was comparable to that of high-density lipoprotein cholesterol. It is interesting that a history of cigarette smoking was associated with lower serum bilirubin and attenuated the protective effect of bilirubin. Another investigation evaluated the relationship of several liver enzymes and bilirubin with coronary artery disease in >800 men (61). A 50% decrease in total bilirubin gave rise to an almost 50% augmentation in the likelihood of developing severe coronary artery disease, whereas there was no association with liver enzymes. In this study, serum bilirubin as an inverse risk factor was roughly equivalent to systolic blood pressure. Levinson (62) also observed an inverse relationship between bilirubin levels and severity of ischemic heart disease in angiographically proven cardiac disease.

A U-shaped relationship, with greater risk of ischemic heart disease at the lowest and highest bilirubin levels, was noted in an 11-year study of >7,000 British men (63). However, when individuals with evidence of liver disease were excluded from the analysis, the relationship between bilirubin levels and protection from atherosclerotic disease was linear (64). Others have also found lower bilirubin levels associated with cigarette smoking, adiposity, and a family history of myocardial infarction (65).

A particularly elegant approach used individuals with Gilbert's syndrome (66), a genetic disorder of impaired bilirubin conjugation causing mild to moderate elevations of unconjugated bilirubin (67, 68). The prevalence of ischemic heart disease in a control population of middle-aged individuals was 12%, compared with 2% in those with Gilbert's syndrome. Elevated bilirubin exerted a more prominent role in protecting from ischemic heart disease than did high-density lipoprotein cholesterol levels. This work was extended to a meta-analysis of 11 studies, finding elevated bilirubin levels associated with diminished risk of atherosclerosis (64). Genetic studies have identified candidate loci for the modest bilirubin elevations associated with protection from cardiac disease (69, 70, 71), including uridine diphosphate glycosyltransferase 1, already known to harbor the mutations of Gilbert's and Crigler-Najjar syndromes.

In peripheral vascular disease, bilirubin levels are lower than in the normal population (72). Atherosclerotic plaques of the carotid arteries predispose to stroke, and in 1,741 subjects who were screened for carotid stenosis, bilirubin levels in the highest quartile were associated with a 32% reduction in risk of developing plaques (73). An animal model of hyperbilirubinemia also is associated with protection against cerebral ischemia. Rats with a mutation in an organic anion transporter manifest hyperbilirubinemia, mimicking the human condition Dubin-Johnson syndrome (74). Stroke damage after middle cerebral artery ligation and reperfusion is diminished in these

animals. An association of serum bilirubin and cancer risk has also been noted. In a cohort of 10,000 Belgian men and women, the risk of cancer mortality declined with elevated serum bilirubin, especially for nonlung cancer (75).

May bilirubin administration be therapeutic? Induction of HO1 by treatment with porphyrin derivatives protects against ischemic insults (76). In perfused rat heart, as little as 100 nanomolar bilirubin reverses the effects of ischemia in cardiac function (77). Intravenous administration of bilirubin ameliorates pulmonary fibrosis induced by bleomycin (78). Injury to liver grafts in rats is prevented by rinsing them with bilirubin (79). Ischemia-reperfusion injury in the rat intestine is ameliorated by intravenous infusion of bilirubin (80, 81).

Conclusions

The combined evidence from animal and human studies indicates that bilirubin is a major physiologic cytoprotectant. A protective action of modest levels of bilirubin does not alter the well-established dangers of kernicterus associated with major elevations of serum bilirubin. Also, it should be noted that many of the studies indicating beneficial effects of bilirubin involve adults, in whom bilirubin disposition may differ markedly from neonates. A substantial number of the studies, however, deal with infants.

One interesting challenge involves linking the clinical studies, which focus on serum levels of bilirubin, with intracellular mechanisms. Serum bilirubin values are 100 to 1,000 times higher than intracellular values. However, ~99% of serum bilirubin is bound to plasma protein and hence unavailable for intracellular antioxidant actions. Because the clinically relevant cellular protection occurs in tissue parenchyma, it is important to understand the way in which serum and intracellular bilirubin levels interrelate. We know little about mechanisms for transporting bilirubin into and out of cells (82, 83). Bilirubin in the serum may have a direct therapeutic action in coping with oxidative stimuli within the bloodstream, such as quenching oxidized low-density lipoprotein (84).

Might there be direct therapeutic implications? In animal studies, bilirubin infusions are therapeutic. Conceivably, one could administer drugs to release bilirubin from its binding sites in serum proteins. Direct translation from animal studies may be feasible. For instance, investigations showing protective effects of bilirubin rinses on organs for tissue grafts in animals (85) may be suitable for application in human subjects. Comparing the antioxidant potential of serum from patients with Gilbert's, Dubin-Johnson, and Rotor syndromes, the last 2 of which have conjugated hyperbilirubinemia, may differentiate the protective effects of unconjugated and conju-

gated bilirubin. Circulating bilirubin may function most predominantly in vascular disease, whereas tissue bilirubin and BVR might be more relevant in diseases of specific organs. Uric acid was once regarded solely as a toxic metabolite responsible for gout, whereas it is now increasingly appreciated as an antioxidant (86). Similarly, physiologic antioxidant roles for bilirubin may detoxify its traditionally nefarious reputation.

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CHAPTER 26

S-Nitrosylated GAPDH Initiates Apoptotic Cell Death by Nuclear Translocation Following Siah1 Binding

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In addition to its well known glycolytic functions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) participates in nuclear events including gene transcription, RNA transport and DNA replication (1). During apoptotic cell death, GAPDH translocates to the nucleus in a number of cell systems (2, 3, 4, 5, 6, 7). Antisense oligonucleotides to GAPDH prevent nuclear translocation of GAPDH and reduce cytotoxicity (2, 3). Molecular events that underlie the nuclear translocation of GAPDH and related cell death have been elusive, because GAPDH lacks a nuclear localization signal (NLS). Moreover, the GAPDH homo-tetramer (with a relative molecular mass of 150,000; M_r 150K), which is required for its proapoptotic actions in the nucleus, is too large to readily enter the nucleus. We now show that cell stressors activate nitric oxide synthase (NOS) leading to S-nitrosylation of GAPDH, which triggers its binding to Siah1, a protein that contains an NLS (8) and mediates the nuclear translocation of GAPDH. In the nucleus, GAPDH stabilizes the rapidly turning over Siah1—enhancing its E3 ubiquitin ligase activity—and causes cell death.

Results

Siah1 Mediates Nuclear Translocation of GAPDH

To seek a potential binding partner that might facilitate GAPDH translocation to the nucleus, we conducted yeast two-hybrid analysis with several portions of GAPDH and a rat hippocampal cDNA library. The full-length bait identifies GAPDH, presumably because of the tendency for this protein to form tetramers (1). The amino-terminal domain identifies huntingtin, previously known to interact with GAPDH (9, 10) as well as an uncharacterized sequence. A bait comprising the carboxy-terminal half of GAPDH identifies Siah1 as the sole interactor out of 10^6 clones. Siah1 is an E3 ubiquitin ligase protein, involved in proteasome-dependent protein degradation (11), and numerous studies indicate a role of Siah1 in apoptosis, including acting as a direct transcriptional target of p53 (12, 13, 14). Siah1, which contains an NLS signal, has nuclear as well as cytosolic localizations (8) and so might mediate GAPDH's nuclear translocation.

Mutational analysis in yeast two-hybrid assays clarifies the binding domains in Siah1 and GAPDH. Binding is critically dependent upon a 19-amino-acid sequence (220–238) in GAPDH with mutation of the single amino acid Lys 225, abolishing binding interactions (Figure 26–1A). We observe *in vitro* binding of GAPDH and Siah1 by utilizing glutathione S-transferase (GST)–Siah1 and GAPDH (Figure 26–1B). To identify protein interactions in intact cells, we transfected HEK293 cells with Myc–Siah1 together with hemagglutinin (HA)–GAPDH or mutant GAPDHs. We observe co-precipitation of GAPDH and Siah1, which is abolished by mutating Lys

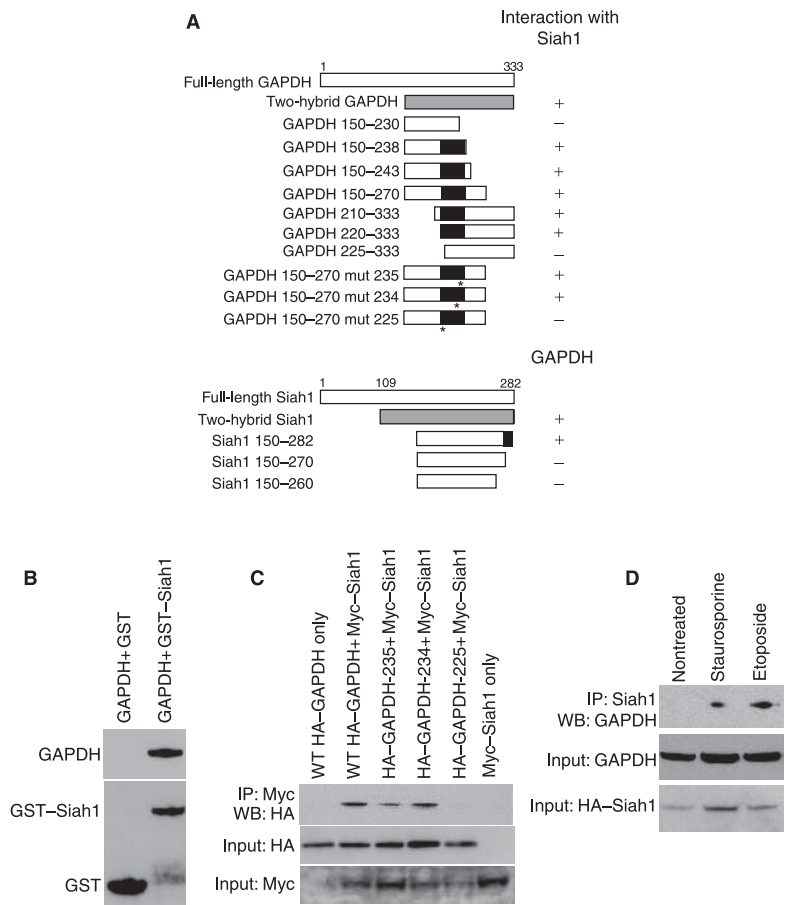


FIGURE 26-1. GAPDH interacts with Siah1.

(A) Mutational analysis of GAPDH–Siah1 interaction. Except as specified, Siah1 constructs from rat were used. Asterisks indicate mutation sites. (B) GAPDH–Siah1 binding *in vitro*. GAPDH was mixed with GST or GST–Siah1 (two-hybrid Siah1: amino acids 109–282 of rat Siah1 open reading frame; compare with (A)). Binding was assessed by GSH-agarose pulldown followed by Western blotting. Representative data from three assays are presented. (C) Co-immunoprecipitation of GAPDH and Siah1 in HEK293 cells. Cell lysates were immunopurified (IP) with anti-Myc antibody and analyzed by Western blotting (WB) with anti-HA antibody. Mutation of Lys 225 abolishes the binding. Representative data from three assays are presented. WT, wild-type. (D) Co-immunoprecipitation of GAPDH and Siah1 in HEK293 cells under stress condition. HEK293 cells were transfected with HA–Siah1. Forty-eight hours after transfection, cells were either nontreated (control), or were treated with 1 μ M staurosporine or 100 μ M etoposide for 24 h. Cell lysates were immunoprecipitated with Siah1 antibody and analyzed by Western blotting with anti-GAPDH antibody. Representative data from three assays are presented.

225 of GAPDH (Figure 26–1C). To examine how cellular stress influences the binding of GAPDH and Siah1, we transfected HA–Siah1 to HEK293 cells and treated them with either staurosporine or etoposide, both of which enhance the binding affinity of GAPDH for Siah1 (Figure 26–1D).

To monitor the influence of Siah1 on the nuclear localization of GAPDH, we transfected N2a cells with Myc–GAPDH alone or together with HA–Siah1 and stained with an anti-Myc antibody (Figure 26–2A). About 10% of cells transfected with GAPDH alone show weak nuclear staining in addition to cytoplasmic staining. By contrast, 70–80% of cells transfected with Siah1 together with GAPDH show nuclear as well as cytosolic staining. The frequency and intensity of nuclear GAPDH staining are markedly decreased in N2a cells transfected with Myc–GAPDH and HA–Siah1 lacking its endogenous NLS (HA–Siah1 Δ NLS). Levels of Siah1 and Siah1 Δ NLS are the same by Western blotting (data not shown). We also assessed the influence of Siah1 on endogenous GAPDH (Figure 26–2B). In most cells transfected with Siah1, endogenous GAPDH is nuclear as well as cytosolic, whereas GAPDH is exclusively cytosolic in cells transfected with Siah1 Δ NLS. We also examined GAPDH–Siah1 disposition by biochemical techniques, transfecting HEK293 cells with HA–Siah1 or HA–Siah1 Δ NLS and purifying the nuclear fractions (Figure 26–2C). In mock-transfected cells, only about 2% of total GAPDH occurs in purified nuclei, whereas transfection with Siah1 causes a 10-fold increase in nuclear GAPDH levels, which is not evident following transfection with Siah1 Δ NLS. When the GAPDH Lys 225 mutant, which does not bind to Siah1 (Figure 26–1A, C), is transfected, Siah1 does not cause the translocation of GAPDH (see Supplementary Information, Figure 26–S1A). Ubiquitination activity of Siah1 is not involved in the nuclear translocation of GAPDH, because Siah1 lacking the RING finger domain, which is responsible for its ubiquitination activity (11), can mediate the nuclear translocation of GAPDH (see Supplementary Information, Figure 26–S1B) and Siah1 does not ubiquitinate GAPDH (see Supplementary Information, Figure 26–S1C). To test the effect of a different isoform of Siah, we transfected HEK293 cells with Siah2, which can also translocate GAPDH to the nucleus (see Supplementary Information, Figure 26–S1D).

GAPDH Stabilizes Siah1

Siah1, a protein with a rapid turnover rate, is degraded by the ubiquitin proteasome system (15) so that under basal conditions tissue levels are extremely low. To examine the influence of GAPDH on Siah1 disposition, we transfected HEK293 cells with Siah1 alone or in combination with various forms of GAPDH, monitoring Siah1 levels in cytosolic and crude nuclear fractions (Figure 26–3A). In the absence of GAPDH, Siah1 levels are barely detectable in the nuclear fraction (P1) and undetectable in the cytosol (S1).

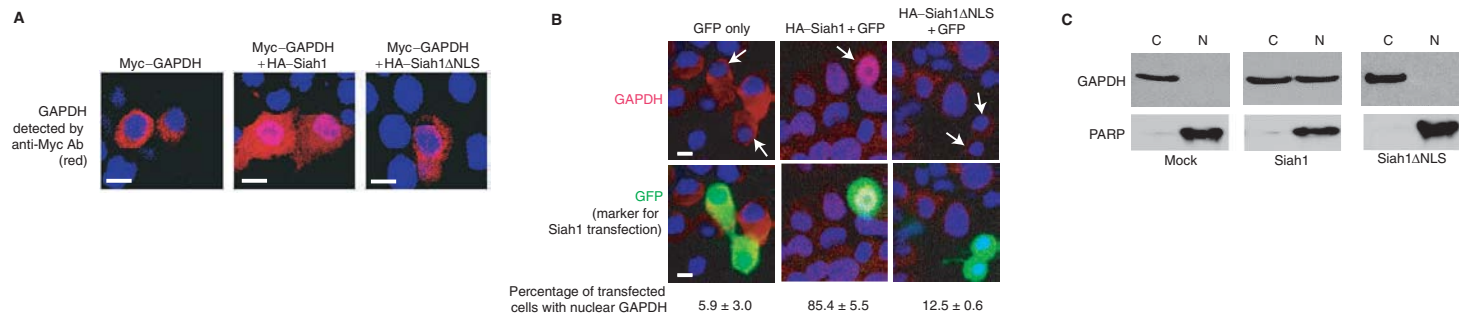


FIGURE 26-2. Nuclear translocation of GAPDH elicited by Siah1.

(A) Immunofluorescent staining for exogenous GAPDH. Constructs of Myc-GAPDH alone, or together with HA-Siah1 or HA-Siah1ΔNLS, were transfected into N2a cells, followed by immunofluorescent staining with anti-Myc antibody (red) and examined by confocal microscopy. The nucleus was stained with Hoechst 33258 (blue). Representative images from five experiments are presented. Scale bar, 20 μm. (B) Immunofluorescent staining for endogenous GAPDH. HA-Siah1, or HA-Siah1ΔNLS was transfected together with GFP into N2a cells, followed by immunofluorescent staining with anti-GAPDH antibody (red) 12 h after transfection, and examined by confocal microscopy. The molar ratio of HA-Siah1 or HA-Siah1ΔNLS construct to GFP construct is 3:1. Because Siah1 turns over rapidly, we used GFP as a marker for transfected cells, indicated by arrows. Values are means ± SD ($n=2$). The nucleus was stained with Hoechst 33258 (blue). Representative pictures from five experiments are presented. Scale bar, 20 μm. (C) Subcellular fractionation: subcellular distribution of GAPDH was examined in cells transfected with Siah1 or Siah1ΔNLS, or in mock-transfected cells. In mock-transfected cells, GAPDH is predominantly cytoplasmic. C, supernatant after 100,000g centrifugation, reflecting cytosol. N, purified nuclei obtained by sucrose gradient centrifugation. PARP was used to confirm identity of the nuclear fraction. Representative data from three assays are presented.

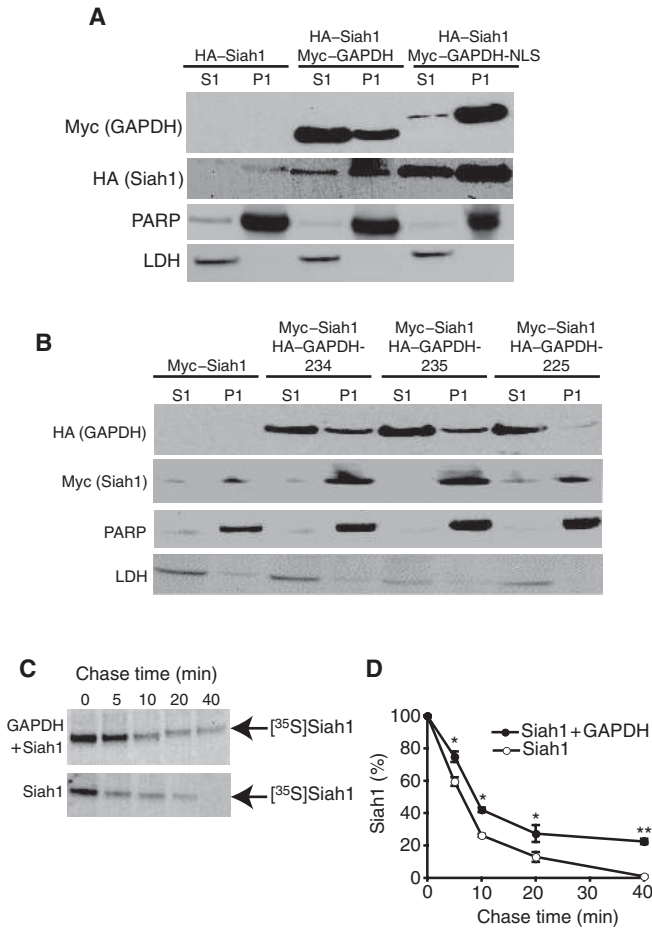


FIGURE 26-3. Stabilization of Siah1 protein by GAPDH.

(A) Levels of Siah1 are augmented by co-transfection with GAPDH and NLS-GAPDH. S1, supernatant after 600g centrifugation, representing a cytosolic fraction. P1, pellet after 600g centrifugation, representing a crude nuclear fraction. Lactate dehydrogenase (LDH) and PARP were used to confirm the cytoplasmic and the nuclear fractions, respectively. Representative data from seven assays are presented. (B) Mutation of GAPDH Lys 225 prevents the GAPDH-induced augmentation of Siah1 levels. Representative data from five assays are presented. (C) Pulse-chase analysis of Siah1 degradation. Constructs of Siah1 with or without GAPDH were transfected into HEK293 cells; transfection was followed by pulse-chase labeling with [35 S]methionine and analysis by immunoprecipitation of Siah1. Representative data from three assays are presented. (D) Quantitative analysis of Siah1 pulse-chase kinetics. Values are a percentage of the amount of Siah1 at time 0 ($n=3$; mean \pm SD; * $P<0.05$ and ** $P<0.05$ versus Siah1). ANOVA statistical analysis indicates that the difference between the degradation curves Siah1+GAPDH and Siah1 is highly significant ($P<0.0005$).

Transfection with GAPDH leads to a threefold increase of Siah1 in P1 and to detectable S1 levels of Siah1. Siah1 concentrations in the nuclear and cytosolic fractions are further augmented by co-transfection of Siah1 and GAPDH containing an exogenous NLS signal (GAPDH-NLS). Augmentation of cytosolic Siah1 presumably reflects Siah1's capacity to shuttle between the cytosol and the nucleus. The ability of GAPDH to augment Siah1 levels requires binding between the two proteins. Thus, mutation of Lys 225 in GAPDH reduces the GAPDH-induced augmentation of Siah1 levels (Figure 26–3B). By contrast, mutations of GAPDH at amino acids 234 or 235, which do not prevent binding to Siah1, have no effect on the levels of Siah1.

To examine the possibility that GAPDH elevates Siah1 levels by slowing its turnover, we conducted a pulse-chase experiment using HEK293 cells (Figure 26–3C, D). In the absence of GAPDH, Siah1 turns over very rapidly with about 50% loss of label in 5 min and no detectable label at 40 min. GAPDH transfection slows Siah1 turnover markedly with substantial levels still evident at 40 min, an effect abolished when GAPDH Lys 225 mutant is transfected (data not shown). The increased levels of Siah1 are unlikely to reflect transcriptional alterations, because we do not observe significant increases in *Siah1* mRNA in cells that are transfected with GAPDH or GAPDH-NLS together with Siah1 (data not shown).

S-Nitrosylation Augments GAPDH–Siah1 Binding

We wondered whether specific molecular alterations of GAPDH trigger its binding to Siah1. We treated HEK293 cells with staurosporine to induce apoptosis and examined gel-purified GAPDH by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure 26–4A). GAPDH mass fingerprints show a mass that is consistent with sulphonation of GAPDH at a single cysteine. Sequence analysis by tandem mass spectrometry (MS/MS) confirms the identity of this substance as GAPDH with the catalytic cysteine (Cys 150) being sulphonated. GAPDH has previously been reported to be covalently linked to NADH *in vitro* (16, 17). Under our experimental conditions we do not detect this modification of the protein (data not shown).

S-Nitrosylation has been established by Stamler et al. (18) as a major signaling mechanism for nitric oxide (NO), and has been shown by them to enhance the binding of procaspase-3 to NOS and acid sphingomyelinase (19). Previously we reported S-nitrosylation of GAPDH in wild-type but not in neuronal NOS (*nNOS*) knockout mice (20), establishing the physiological relevance of this modification. Sulphonation of cysteine can arise following S-nitrosylation (–SNO) via hydrolysis to sulphenic acid (–SOH) and sequential oxidation to sulphinic acid (–SO₂H), and then sulphonic acid (–SO₃H) (21). To determine whether S-nitrosylation elicits nuclear translocation of

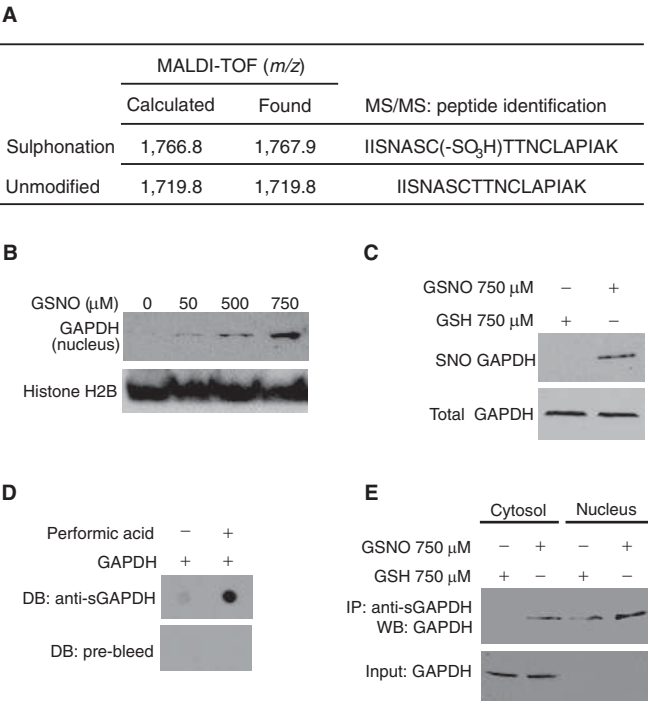


FIGURE 26-4. NO causes nuclear accumulation of sulphonated GAPDH (sGAPDH).

(A) Apoptosis elicits sulphonation of GAPDH at Cys 150. HEK293 cells were treated with 1 μM staurosporine. GAPDH was immunopurified from a detergent-insoluble fraction and resolved on SDS-PAGE. In-gel-digested GAPDH was analyzed by MALDI-TOF mass spectrometry. HPLC-purified fragments were used to identify peptide sequence in MS/MS mass spectrometry (-SO₃H, sulphonic acid derivative). The catalytic cysteine is Cys 150 for rat, and Cys 152 for human GAPDH open reading frame. (B) GAPDH translocates to the nucleus in a GSNO dose-dependent manner. HEK293 cells were treated with GSNO using indicated concentrations for 24 h. Cells were fractionated to cytosol and nuclear fractions. (C) S-Nitrosylation of GAPDH in HEK293 cells. HEK293 cells were treated with either 750 μM GSH or 750 μM GSNO for 24 h. Cell lysates were subjected to the biotin switch assay (SNO GAPDH, S-nitrosylated GAPDH). (D) Anti-sGAPDH antibody detects sulphonic acid derivatives of GAPDH. GAPDH was treated with 1 mM performic acid or mock treated, and analyzed by dot blotting (DB). (E) GSNO causes sGAPDH accumulation in the nucleus. Cells were treated as described in (C). Cytosol and nuclear fractions were immunopurified with anti-sGAPDH antibody and analyzed by Western blotting with anti-GAPDH antibody. Overexposure of the input Western blot shows GAPDH in the nucleus as shown in (B). These results were replicated twice.

GAPDH, we treated HEK293 cells with *S*-nitroso-glutathione (GSNO), an NO donor. Subcellular fractionation reveals nuclear translocation of GAPDH in response to GSNO in a concentration-dependent manner (Figure 26–4B). Siah1 protein levels are augmented by GSNO treatment (see Supplementary Information, Figure 26–S1E). Using the biotin switch assay (20), we confirm *S*-nitrosylation of GAPDH by GSNO, but not by glutathione (GSH) (Figure 26–4C). To quantify sulphonated GAPDH, we produced an antibody to a sulphonated GAPDH (sGAPDH), which contains cysteic acid ($-\text{SO}_3\text{H}$) at Cys 150 instead of cysteine ($-\text{SH}$). GAPDH, treated with performic acid to transform cysteines into their sulphonated derivatives, provides immunoreactivity to the anti-sGAPDH antibody (Figure 26–4D). Following GSNO treatment, we detect sGAPDH in the nucleus (Figure 26–4E).

Because sulphonation can derive from *S*-nitrosylation of GAPDH, we wondered whether *S*-nitrosylation enables the binding of GAPDH to Siah1, and their nuclear translocation following apoptotic stimuli. Treatment of GAPDH with GSNO leads to substantial GAPDH–Siah1 binding (Figure 26–5A, B). Prolonged exposure reveals binding of untreated GAPDH to Siah1 (Figure 26–1B). The action of GSNO reflects its NO donation. Thus, sodium nitroprusside (SNP), another NO donor, elicits the same augmentation of GAPDH–Siah1 binding (data not shown). Binding of GAPDH to Siah1 in the presence of GSNO depends on *S*-nitrosylation, because dithiothreitol (DTT), which reduces nitrosothiols, abolishes GAPDH–Siah1 binding (Figure 26–5C). Ascorbate, an intracellular reducing agent, also reverses GAPDH–Siah1 binding (data not shown). The binding of *S*-nitrosylated GAPDH to Siah1 requires modification at Cys 150, because mutation to serine of Cys 150 in GAPDH abolishes the effect of GSNO on the binding (Figure 26–5D). To ascertain the selectivity of *S*-nitrosylation, we compared influences of the same concentrations of GSNO and other oxidative agents, hydrogen peroxide and peroxynitrite (Figure 26–5E). We observed less binding to Siah1 of GAPDH treated with hydrogen peroxide or peroxynitrite than with GSNO.

Recently, Bowtell et al. identified a Siah-binding motif (22). We detect a similar motif near Lys 225 of GAPDH. Moreover, a peptide derived from this region inhibits the binding of *S*-nitrosylated GAPDH and Siah1 (see Supplementary Information, Figure 26–S1F, G, H).

To assess the influence of NO on the binding of GAPDH and Siah1 in intact cells, we transfected Myc–Siah1 into HEK293 cells, which were then treated with SNP. Using the biotin switch assay (20), we confirm *S*-nitrosylation of GAPDH by SNP (Figure 26–5F). Co-immunoprecipitation experiments reveal increased binding of GAPDH to Siah1 following SNP treatment (Figure 26–5F).

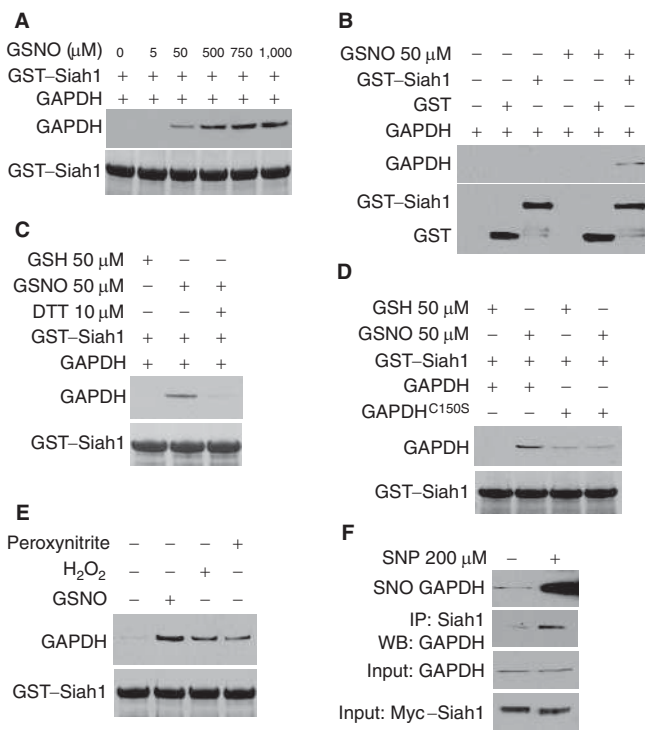


FIGURE 26-5. S-nitrosylation of GAPDH enhances its binding to Siah1.

(A) NO increases the binding of GAPDH to Siah1 in a concentration-dependent manner. GAPDH was pre-treated with GSNO at different concentrations as indicated. GST-Siah1 (two-hybrid Siah1: amino acids 109– 282 of rat Siah1 open reading frame; compare with Figure 26-1A) was added, and binding was assessed by GSH-agarose pulldown followed by Western blotting. (B–E) Binding reactions were performed as described in (A). (B) NO increases the binding of GAPDH to Siah. GAPDH was pre-treated with GSNO or mock treated, and GST or GST-Siah1 was added. (C) DTT abolishes the effect of NO on the binding of GAPDH to Siah1. GAPDH was exposed to DTT for 10 min at 37°C after the GSNO treatment. (D) GAPDH Cys 150 mutation abolishes the effect of NO on the binding of GAPDH to Siah1. Recombinant His-GAPDH or His-GAPDH^{C150S} was used in the binding reactions. There is some basal binding of C150S, which may reflect the chemical resemblance between serine (-OH) and sulphenic acid (-SOH), which also enhances GAPDH's binding to Siah1 as in (E). (E) Siah1 preferentially binds GSNO-treated GAPDH compared with hydrogen peroxide (H₂O₂) or peroxyntirite-treated GAPDH. Each chemical was used in the binding reactions at 50 μM concentration. (F) NO enhances the binding of GAPDH to Siah in intact cells. HEK293 cells were transfected with Myc-Siah1. After incubation with MG132 for 4 h, 200 μM SNP were added, and cells were further incubated for 1 h. Cell lysates were subjected to the biotin switch assay and immunoprecipitation with anti-Siah1 antibody. These results were replicated at least three times.

S-Nitrosylation of GAPDH Mediates Nuclear Translocation of GAPDH–Siah1 and Cell Death

To ascertain the physiological relevance of NO-mediated binding of GAPDH to Siah1, we used a macrophage cell line, RAW264.7. Treatment of RAW264.7 cells with the endotoxin constituent lipopolysaccharide (LPS) leads to induction of inducible NOS (iNOS) and apoptotic cell death within 24 h, at which time there is no evidence of necrosis (23). At 24 h after LPS treatment, we observe S-nitrosylation of GAPDH and increased binding of GAPDH and Siah1, both of which are prevented by the iNOS inhibitor N-(3-(aminomethyl)benzyl)acetamidine (1400W) (Figure 26–6A, B). Subcellular fractionation shows that GAPDH is translocated to the nucleus in response to LPS, an effect that is reversed by an iNOS inhibitor (Figure 26–6C). Densitometry analysis reveals that $4.3 \pm 0.8\%$ ($n=3$) of GAPDH translocates during this process. Immunocytochemical examination also establishes nuclear translocation of GAPDH following LPS treatment (Figure 26–6D).

Thus, LPS induces iNOS, leading to S-nitrosylation of GAPDH, which enables GAPDH to bind to Siah1 and translocate to the nucleus. To ascertain the importance of GAPDH for apoptotic cell death, we depleted it by small interfering RNA (siRNA) with pyruvate-containing medium to compensate for any requirement of GAPDH in the glycolytic pathway (24). siRNA treatment depletes GAPDH as assessed by Western blotting (Figure 26–6E). The apoptotic action of LPS and interferon- γ (IFN- γ) (23) is abolished by the iNOS inhibitor 1400W or siRNA treatment at 24 h (Figure 26–6F). The influence of siRNA treatment is unrelated to the formation of NO, detected by its oxidized product nitrite (NO_2^-), which is the same in the presence or absence of siRNA. In a separate experiment, LPS-elicited apoptosis is prevented by antisense but not sense oligonucleotides to GAPDH (see Supplementary Information, Figure 26–S2A). These data indicate that the NO–S-nitrosylation–GAPDH–Siah1 cascade initiates apoptotic cell death in RAW264.7 cells.

Because Cys 150 is the catalytic site of GAPDH, we wondered whether overall cellular glycolytic activity is influenced by Cys 150-S-nitrosylation. We do not observe a significant decrease in GAPDH glycolytic activity until 72 h after LPS treatment (see Supplementary Information, Figure 26–S2B). The decreased activity at that time corresponds to increased trypan-blue-positive cells (data not shown), indicative of cell death that may be necrotic, which might arise in response to a bioenergetic crisis (see Supplementary Information, Figure 26–S2B).

For a more physiological preparation, we utilized peritoneal macrophages that were isolated from wild-type and iNOS knockout mice. Induction of iNOS with LPS leads to S-nitrosylation of GAPDH in wild-type but

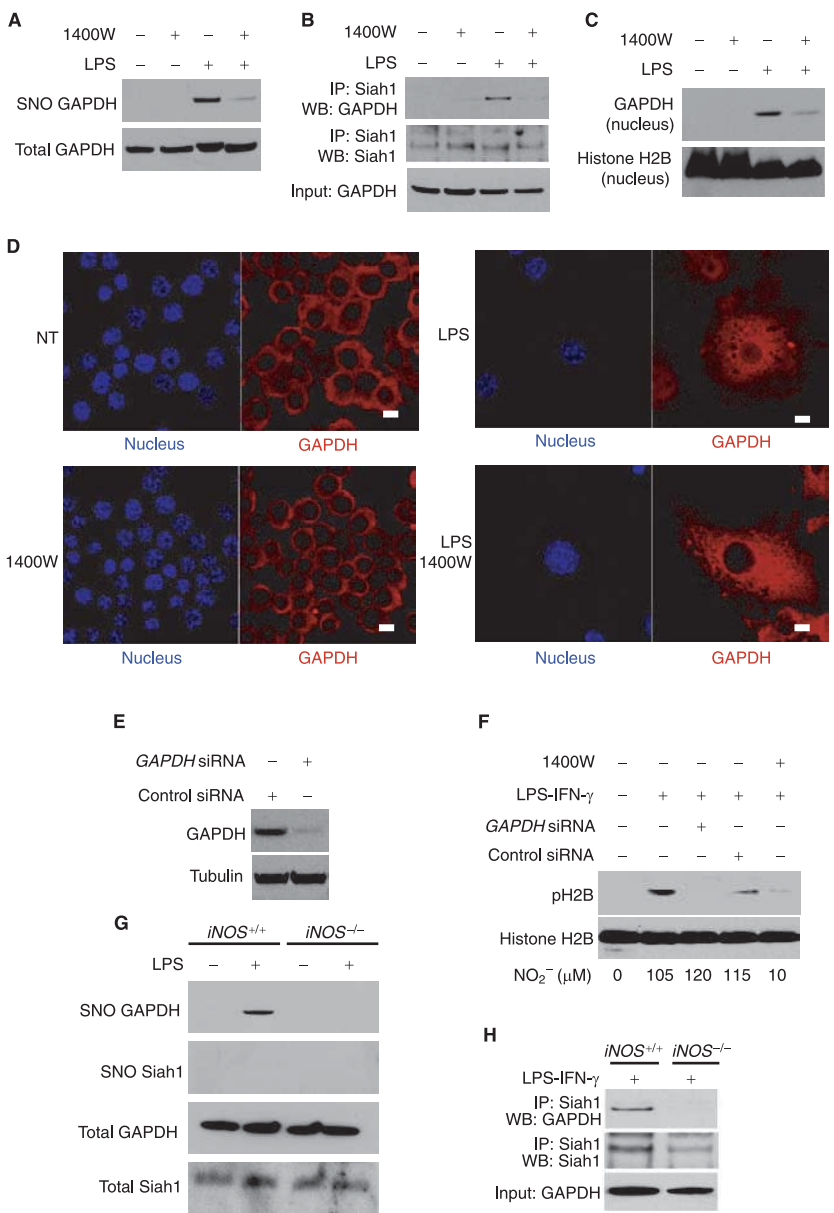


FIGURE 26-6. NO promotes translocation of GAPDH to the nucleus following Siah1 binding.

(A) NO generated from iNOS causes S-nitrosylation of GAPDH. RAW264.7 cells were either nontreated, treated with 1400W (100 μM), stimulated with LPS for 24 h, or pre-treated with 1400W for 1 h then stimulated with LPS for 24 h. Cell lysates were subjected to the biotin switch assay. (B–D) RAW264.7 cells were treated as in (A).

FIGURE 26-6. NO promotes translocation of GAPDH to the nucleus following Siah1 binding (*continued*).

(B) NO increases binding of GAPDH to Siah1. Cell lysates were immunoprecipitated with anti-Siah1 antibody. (C) NO promotes translocation of GAPDH to the nucleus. Nuclear fractions were analyzed by Western blotting. (D) GAPDH translocates to the nucleus upon LPS stimulation. RAW264.7 cells were immunostained with anti-GAPDH antibody and examined by confocal microscopy. The nucleus was visualized with DAPI. Scale bar, 10 μm . (E) siRNA to GAPDH depletes GAPDH protein in RAW264.7 cells. (F) siRNA to GAPDH prevents apoptosis in RAW264.7 cells. RAW264.7 cells were transfected with siRNA to GAPDH or control, and stimulated with LPS and IFN- γ . Cell lysates were analyzed by Western blotting for phosphorylated histone H2B (pH2B), an apoptotic marker (48). NO_2^- concentration was measured by the Griess reagent ($n=2$). In a similar experiment, we observe that caspase-3 is cleaved following treatment with LPS-IFN- γ and that this effect is reduced by siRNA to GAPDH but not by control siRNA (data not shown). (G) NO causes S-nitrosylation of GAPDH in primary macrophages. Peritoneal macrophages from wild-type and iNOS knockout mice were stimulated with LPS for 24 h. Cell lysates were subjected to the biotin switch assay. (H) NO increases the binding of GAPDH to Siah1 in primary macrophages. Peritoneal macrophages were stimulated with LPS and IFN- γ for 24 h. Cell lysates were immunoprecipitated with anti-Siah1 antibody. These results were replicated at least three times.

not knockout mice, indicating the physiological relevance of S-nitrosylation (Figure 26-6G). Siah1 is not S-nitrosylated either in wild-type or iNOS knockout mice, implying the specificity of the modification (Figure 26-6G). Binding of GAPDH and Siah1 is decreased in iNOS knockout mice as assessed by co-immunoprecipitation (Figure 26-6H). Consequently, nuclear GAPDH and cell death are decreased in iNOS knockout macrophages (see Supplementary Information, Figure 26-S2C, D).

Roeder et al. (24) identified GAPDH as a component of a coactivator for Oct-1 during S phase in HeLa cells. To examine whether binding of GAPDH to Siah1 mediates the nuclear translocation of GAPDH in S phase, we transfected the GAPDH Lys 225 mutant, which can be S-nitrosylated (see Supplementary Information, Figure 26-S3A) but does not bind to Siah1 (Figure 26-1C). The mutant translocates to the nucleus in S phase (see Supplementary Information, Figure 26-S3B). During S phase, GAPDH is not S-nitrosylated (data not shown) and does not reside in the nuclear matrix, whereas with apoptotic stress GAPDH localizes to the nuclear matrix (see Supplementary Information, Figure 26-S3C). Thus, nuclear translocation of GAPDH during S phase is presumably Siah1-independent and reflects a process that is distinct from nuclear translocation during apoptosis.

Neuronal cell death in the brain following glutamate excitotoxicity has been associated with excess production of NO by nNOS, because neurotox-

icity is diminished in pure neuronal cultures by NOS inhibitors (25) and in *nNOS* knockout mice (26). To examine the role of *nNOS* in regulating GAPDH–Siah1 interactions, we utilized cerebellar granule neurons from wild-type and *nNOS* knockout mice. To activate *nNOS*, we treated preparations with the glutamate derivative NMDA (*N*-methyl-D-aspartate), which elicits a substantial augmentation in binding of GAPDH to Siah1 that is virtually abolished in *nNOS* knockout mice (Figure 26–7A). To assess the influence of GAPDH and Siah on cell viability, we transfected plasmids expressing small hairpin RNA (shRNA) to deplete GAPDH and Siah in cerebellar granule neurons. The RNAi treatment depletes GAPDH and Siah as assessed by Western blotting for GAPDH and Siah (see Supplementary Information, Figure 26–S4A). RNAi to Siah alone does not influence GAPDH (Figure 26–7B). Neuronal cell death elicited by NMDA is prevented by RNAi to Siah or GAPDH (Figure 26–7C), and the effect is RNAi-concentration-dependent (see Supplementary Information, Figure 26–S4B). Similarly, neuronal death elicited by GSNO or etoposide is prevented by RNAi to GAPDH and Siah (see Supplementary Information, Figure 26–S4C).

Siah1's Cytotoxic Actions Involve Ubiquitination and Degradation of Nuclear Proteins

Siah is an E3 ubiquitin ligase with diverse substrates including nuclear proteins, cytosolic proteins, and membrane receptors (11). The E3 ubiquitin ligase activity of Siah is critically dependent upon its RING finger domain (11). To ascertain whether ubiquitin-mediated nuclear protein degradation by Siah accounts for GAPDH-related cell death, we used mutant Siah1 lacking the RING finger domain or the NLS (Figure 26–8A). Consistent with Siah's known pro-apoptotic actions (12, 13), about 20% of cells die following Siah1 transfection, whereas cytotoxicity is doubled by co-transfection of GAPDH with Siah1. Co-transfection of Siah1 with GAPDH-NLS leads to a further augmentation of the cytotoxicity, indicating the importance of GAPDH's nuclear localization for cytotoxicity. The cytotoxicity is correlated with the extent of Siah1 stabilization. Transfection of Siah1ΔNLS, which maintains ubiquitination activity (see Supplementary Information, Figure 26–S1B), elicits much less cytotoxicity than native Siah1. Transfection of Siah1 lacking the RING finger domain (Siah1ΔRING) abolishes its cytotoxic actions. By contrast, Siah1 lacking the 24 amino acids to the N-terminal side of the RING finger domain retains cytotoxic activity. Consistent with these results, treatment with a proteasome inhibitor, MG132, diminishes cytotoxicity (data not shown).

Siah can degrade a variety of nuclear proteins (11). As a reflection of Siah's nuclear actions, we examined one of these, nuclear receptor corepressor (N-CoR). Degradation of N-CoR was assessed by chloramphenicol acetyltransferase (CAT) activity driven by a Gal4-containing construct

fused to N-CoR (27, 28). In HEK293 cells, co-transfection with Siah1 inhibits N-CoR activity as reflected by augmented CAT activity (Figure 26–8B). A further increase in CAT activity occurs with the addition of GAPDH to Siah1, whereas GAPDH alone does not influence CAT activity. N-CoR protein levels are depleted by co-transfection with Siah1 and GAPDH, whereas Siah1 transfection alone diminishes N-CoR. Thus, GAPDH markedly augments the ability of Siah1 to degrade nuclear targets.

Discussion

Our findings establish a cell death cascade whereby cell stressors activate NOS, leading to *S*-nitrosylation of GAPDH, which activates its binding to Siah1 followed by nuclear translocation (Figure 26–8C). GAPDH stabilizes the rapidly turning over Siah1, augmenting its nuclear levels, facilitating degradation of nuclear targets, and eliciting cell death. Our findings indicate that NO formed from iNOS may be a general initiator of this process, because iNOS is one of biology's most inducible and ubiquitous enzymes. With GAPDH as a sensor, iNOS may function as a universal regulator of cell death. In neuronal tissues, stimulation of nNOS by influences such as glutamate-NMDA receptor activation may also initiate GAPDH–Siah1 binding and mediate neurotoxicity elicited by NMDA–nNOS signaling (25). Cell stress causes the formation of several reactive oxygen species (29). Hydrogen peroxide treatment of GAPDH elicits GAPDH–Siah1 binding but is substantially less effective than *S*-nitrosylation. Reactive oxygen species and NO may interact in protein *S*-nitrosylation, as reported for superoxide (18). In the present study, we used staurosporine or etoposide as stressors to activate GAPDH–Siah1 signaling, because their toxic actions involve NO (30, 31).

Moncada et al. (32, 33) established a role for NO in cell death by impairing cytochrome *c* oxidase's actions in the mitochondrial respiratory chain. This defect in cytochrome *c* oxidase leads to disruption of the mitochondrial membrane potential, a key trigger for cell death, which can be delayed by supplementation with glycolytically derived ATP (32, 33). Our proposed model of NO-induced death signaling is complementary to the pathway proposed by Moncada et al. (32, 33). In our experimental model, during the first 24 h following cell stress, a small portion of GAPDH is *S*-nitrosylated and translocated to the nucleus with negligible effects on overall cellular glycolysis. Thus, the GAPDH–Siah1 mechanism of NO-induced apoptotic cell death is independent of glycolytic impairment. Impairment of glycolysis seems to participate primarily in later stages of cell death, which may be necrotic and triggered by the initial GAPDH–Siah1 phase.

Excess production of NO has been implicated in multiple types of cell death (25, 26, 34). One mechanism involves DNA damage by NO leading to

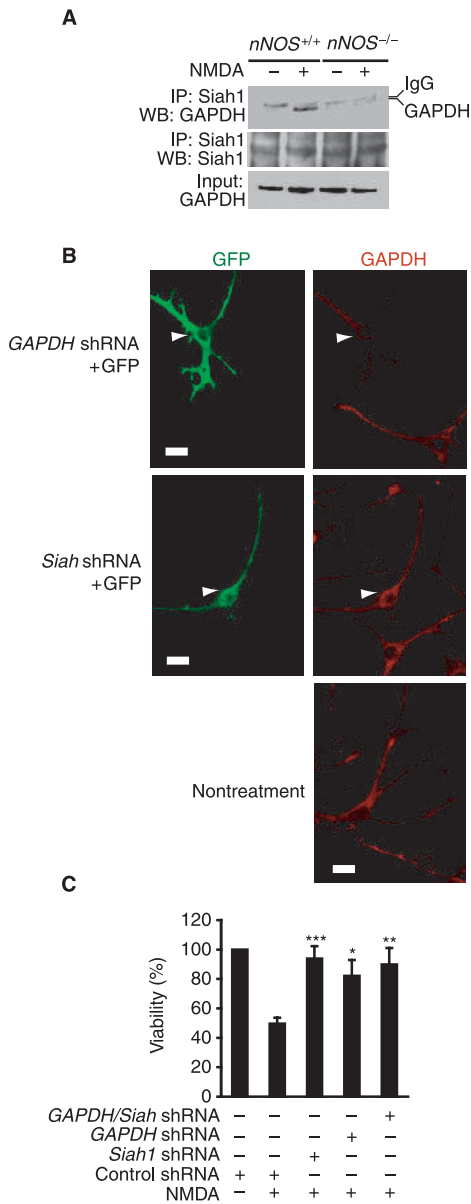


FIGURE 26-7. GAPDH-Siah1 cell death cascade in neurons.

(A) Endogenous NO increases the binding of GAPDH to Siah1 in primary neurons. Primary cerebellar granule neurons from wild-type and *nNOS* knockout mice were treated with 300 μ M NMDA (49) for 10 min and incubated for 24 h. Cell lysates were subjected to immunoprecipitation with anti-Siah1 antibody. (B) Suppression of endogenous GAPDH by shRNA in primary granule neurons. Plasmid constructs expressing

FIGURE 26-7. GAPDH-Siah1 cell death cascade in neurons (continued).

GAPDH shRNA or Siah shRNA (*Siah1* shRNA+*Siah2* shRNA) were co-transfected with GFP. Representative images are depicted 2 days after transfection with a fluorescence microscope. White arrowheads indicate transfected cells. Scale bar, 10 μ m. These results were replicated twice. (C) shRNA to GAPDH and Siah prevents NMDA-caused neuronal death. Primary cerebellar granule neurons were transfected with plasmid expressing shRNA to control ($n=5$), GAPDH ($n=2$), *Siah1* ($n=4$), or a combination of GAPDH, *Siah1*, and *Siah2* ($n=5$) (*Siah* shRNA: *Siah1* shRNA+*Siah2* shRNA). Transfected cells were treated as in (A). Viability was assessed by GFP staining (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus NMDA+control shRNA).

activation of poly ADP-ribose polymerase (PARP) with depletion of NAD⁺ and ATP, leading to energy depletion (35). Alternatively, NO combined with superoxide can form peroxynitrite, a powerful oxidant that promotes DNA breaks, lipid peroxidation, and protein oxidation (36). These actions of NO involve extensive cell damage and necrotic cell death. By contrast, the NO-GAPDH-Siah1 pathway utilizes proteasome-dependent degradation of Siah1 substrates to cause apoptotic cell death. Nuclear translocation of GAPDH occurs by 3 h following apoptotic stressors (2) at a time that ATP levels are normal (37), because ATP is required for the energy demands of apoptosis. This contrasts with ATP depletion, which is associated with necrosis or later stages of apoptosis.

There are three murine Siah genes: *Siah1a*, *Siah1b*, and *Siah2* (38). The human and rat genomes contain two Siah family genes, *SIAH1* and *SIAH2*, encoding proteins that are almost identical to murine *Siah1a* and *Siah2*, respectively (39). In our study, both *Siah1* and *Siah2* can shuttle GAPDH to the nucleus. Although we show that N-CoR is one of *Siah1*'s substrates that is degraded in the nucleus, our experiments do not establish that N-CoR is principally responsible for the action of *Siah1* on apoptosis. As *Siah1* affects multiple targets in the nucleus, events downstream of the GAPDH-Siah1 cascade remain to be clarified. Matsuzawa and Reed (40) identified a role of Siah in phosphorylation-independent β -catenin degradation, during which Siah-interacting protein (SIP) mediates the effects of *Siah1* on β -catenin protein levels. In a similar manner, GAPDH may enable *Siah1*'s targeting of specific substrates to initiate cell death.

A role for GAPDH in cell death may have therapeutic relevance, especially in neurodegenerative diseases. Recently, a genetic association between the GAPDH locus on chromosome 12 and late-onset Alzheimer's disease was reported (41). (-)-Deprenyl, a monoamine oxidase B (MAO-B) inhibitor, is neuroprotective via mechanisms that do not seem to involve inhibition of MAO-B (42). (-)-Deprenyl and its derivative TCH346, which lacks MAO-B inhibitory activity but is comparably neuroprotective (6, 43), bind to

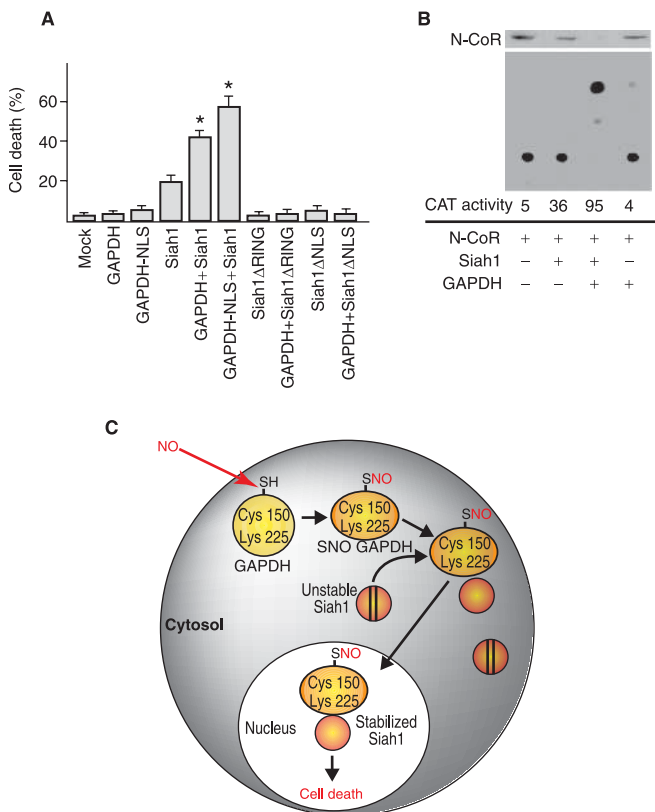


FIGURE 26-8. Siah1’s cytotoxic actions involve degradation of its nuclear substrates.

(A) Cytotoxicity elicited by GAPDH and Siah1 requires nuclear localization of GAPDH and Siah1’s RING finger domain. Various forms of GAPDH and Siah1 constructs were transfected into HEK293 cells. Cytotoxicity was evaluated by LDH assay 3 days after transfection. Transfection of GAPDH, or GAPDH with exogenous NLS signal (GAPDH-NLS) is nontoxic. By contrast, 20% of cells transfected with Siah1 die 3 days after transfection. Co-transfection of GAPDH or GAPDH-NLS together with Siah1 augments cytotoxicity twofold and almost threefold, respectively (* $P < 0.001$). Transfection of Siah1ΔRING does not elicit toxicity, even following co-transfection with GAPDH. Transfection with Siah1ΔNLS leads to markedly reduced toxicity, even following co-transfection with GAPDH. Similar results were obtained by a caspase-3 cleavage assay (data not shown). (B) GAPDH–Siah1 degrades nuclear proteins. N-CoR degradation was monitored by CAT assay using Gal4–N-CoR fusion protein, which was analyzed by Western blotting. Transcriptional repression mediated by a Gal4–N-CoR fusion protein is partially prevented by co-transfection of Siah1, whereas co-transfection of GAPDH and Siah1 almost completely prevents the repression by N-CoR. Co-transfection of GAPDH alone does not affect transcriptional repression. The percentage conversion of CAT is indicated below the lanes. Representative data

FIGURE 26-8. Siah1's cytotoxic actions involve degradation of its nuclear substrates (continued).

from five assays are presented. (C) Schematic diagram of NO-S-nitrosylation-GAPDH-Siah1 cell death cascade. NO causes S-nitrosylation of GAPDH at Cys 150. S-nitrosylation of GAPDH augments its binding to Siah1 at Lys 225. NLS of Siah1 mediates nuclear translocation of GAPDH. GAPDH stabilizes Siah1, facilitating its degradation of nuclear proteins, which leads to cell death.

GAPDH (5) and prevent nuclear translocation of GAPDH (6). Agents that inhibit GAPDH-Siah1 interactions may provide a useful approach to cytoprotective therapy.

Methods

Reagents. Unless otherwise noted, chemicals were purchased from Sigma (St. Louis, MO). Protein G agarose and protein A agarose were purchased from Oncogene (Cambridge, MA). 1400W was purchased from Alexis (San Diego, CA). Two methods for RNA interference were used in this study. Synthesized small oligonucleotides for siRNA were from Dharmacon RNA Technologies (Lafayette, CA). The vector system for shRNA is as described (44, 45). The following sequences were chosen for silencing the gene expressions: GAPDH, 5'-CGGGAAGCTCACTGGCATG-3'; Siah1, 5'-TGTAAC-TATTTCCATGTGT-3'; Siah2, 5'-CTTTGCCTACAGACTGGAG-3'; and control, 5'-AACACAGCUAUCGAGCCC-3'. Antibodies were obtained from the following companies: anti-GAPDH monoclonal antibody was from Biogenesis (Poole, UK); anti-histone H2B antibody, anti-phospho-histone H2B, and anti-N-CoR antibody were from Upstate (Lake Placid, NY); anti-HA antibody was from Babco (Berkeley, CA); anti-Myc antibody was from Calbiochem (San Diego, CA); and anti-Siah1 antibody was from Santa Cruz (Santa Cruz, CA). Polyclonal antibody against GAPDH was prepared as follows: GST-tagged GAPDH was produced in *Escherichia coli*, purified through glutathione-Sepharose beads (Pharmacia Biotech, Piscataway, NJ), and used for antigen. Antisera were produced by a service of Research Genetics, and the sera were immunopurified. The immunogen sulphonated GAPDH peptide (SNASC(-SO₃H)TTNCLAPLAKV) and a control peptide (SNASCTTNCLAPLAKV) were produced by American Peptide Company (Sunnyvale, CA), and polyclonal rabbit antibody was generated by Lampire Biological Laboratories (Pipersville, PA).

Plasmid Construction. GAPDH was cloned from a rat cDNA library (Clontech, Palo Alto, CA) using the following primers: 5'-GGATCCGTCGAC-GATGGTGAAGGTCGGTGTCAAC-3' and 5'-AAGATAAGGAATGCGGCCGCAGGGGTTTCTTACTCCTTGG-3'. GAPDH^{C150S} was produced using the following primers: 5'-CAATGCATCCTCCACCACCAAC-3' and 5'-GTTGGTGGTGGAGGATGCATTG-3'. Deletion mutants for yeast two-hybrid were produced by PCR with newly synthesized primers corresponding to the amino acids described in Figure 26-1A. The mutants with one amino-acid substitution for yeast two-hybrid were produced as described. An

expression vector with NLS sequence (Invitrogen, Carlsbad, CA) was used for constructing GAPDH-NLS plasmid. The deletion mutants of Siah1 Δ NLS and Siah1 Δ RING were produced as described using the following internal primers: 5'-TCCTCCAGCAGCAGCATGACCATTCAGCTCAAGTC-3' for antisense of Siah1 Δ NLS, 5'-GCTGCTGCTGGAGGATTGACTTGGGAAGCCAC-3' for sense of Siah1 Δ NLS, 5'-GCGAATGGATCCCAACTGAAGAATAGGTGGCAATAC-3' for antisense of Siah1 Δ RING, and 5'-CCACCTATTCTTCAGTTGGGATCCATTCGCAACTTG-3' for sense of Siah1 Δ RING. N-CoR-Gal4 plasmid was produced by N-CoR sequences corresponding to its 1–160 amino acids into an expression vector with Gal4.

Yeast Two-Hybrid Analysis. The full-length, N-terminal (amino acids 1–150) and C-terminal portions (amino acids 149–333) of rat GAPDH open reading frame were cloned into yeast expression vector pPC97, containing the *Gal4* DNA binding domain. These constructs were used for the screening of a rat hippocampal cDNA library cloned into pPC86, containing the Gal4 transactivation domain.

Cloning of Rat Full-Length Siah1 cDNA. An adult rat brain cDNA library in lambda ZAPII vector (Stratagene, La Jolla, CA) was screened using the yeast two-hybrid Siah1 fragment, labeled with [³²P]dCTP using a random priming DNA labeling kit (Roche, Indianapolis, IN). A total of 1×10^6 clones were screened, yielding 35 overlapping clones.

Mass Spectrometry. HEK293 cells were treated with staurosporine (1 μ M) for 24 h. Cells were lysed in RIPA A (0.3% Triton X-100, 50 mM Tris pH 7.4, and 1 mM EDTA) with rotation at 4°C for 30 min. Cell lysates were centrifuged at 14,000g for 10 min. GAPDH was extracted from the pellet with RIPA B (1% Triton X-100, 1% SDS, 50 mM Tris pH 7.4, 500 mM NaCl, and 1 mM EDTA), affinity precipitated with anti-GAPDH antibody, subjected to SDS polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing conditions, and visualized by colloidal Coomassie staining. Gel-purified GAPDH was tryptic digested and analyzed by MALDI-TOF mass spectrometry (Voyager DESTR, Applied Biosystems, Foster City, CA). Peptide identity was confirmed by liquid chromatography tandem mass spectrometry coupled with electrospray ionization and fragmentation by collision-induced dissociation (QSTAR, Applied Biosystems, Foster City, CA) in the mass spectrometry facility at Johns Hopkins University School of Medicine.

GST Pulldown Assay. GST-tagged proteins were prepared according to the manufacturer's recommendations (Amersham Biosciences, Piscataway, NJ) and purified through glutathione-Sepharose (Amersham Biosciences). His-tagged proteins were prepared and purified using TALON metal affinity resins (Clontech). Five micrograms GAPDH were incubated with nitric oxide or reactive oxygen donors for 30 min at 37°C in 1 mL binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1 mg mL⁻¹ BSA, 50 μ M NAD⁺, 10 mM sodium phosphate, and 50 μ M glyceraldehyde-3-phosphate). Thirty microliters of 50% GST–Siah1–glutathione-Sepharose or GST–glutathione-Sepharose were added to the mixture, and incubated for 15 min at room temperature with ro-

tation. The beads were washed four times with 1 mL binding buffer and separated by SDS-PAGE, and analyzed by Western blotting.

Co-immunoprecipitation. Cells were lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% CHAPS, 100 μ M deferoxamine, and 1 mM EDTA) and homogenized by passing through a 26-gauge needle. Crude lysates were cleared of insoluble debris by centrifugation at 14,000g. IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% CHAPS, 100 μ M deferoxamine, 1 mM EDTA, and 0.1 mg mL⁻¹ BSA) was added to 100 μ g of cell lysates to bring samples to a total volume of 1,000 μ L. Anti-Siah1 antibody and 30 μ L of protein G agarose were added and incubated on a rotator at 4°C. The protein G agarose was washed four times with lysis buffer and quenched with 30 μ L of SDS sample buffer. Co-immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting.

Subcellular Fractionation. Subcellular fractionation using a sucrose gradient was performed as described (2), with minor modifications. The P1 fraction was obtained by a 10-min centrifugation at 600g, after which purified nuclei were obtained by centrifugation over a 2 M sucrose cushion at 100,000g for 1 h. The nuclear matrix was prepared from HeLa cells, according to the established protocol (46).

Cell Culture. RAW264.7, HEK293, and N2a cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 2 mM L-glutamine at 37°C with a 5% CO₂ atmosphere in a humidified incubator. Peritoneal macrophages were elicited by intraperitoneal injection of 1.5 mL of 3% sterile thioglycolate medium. After 4 days, mice were killed and macrophages were collected as described (47). To stimulate macrophages, LPS (1 μ g mL⁻¹) with or without IFN- γ (10 ng mL⁻¹) were added to medium. For RNAi experiments, media for RAW264.7 cells was supplemented with 1 mM pyruvate, and cells were transfected with 2 μ g of GAPDH or control siRNA using TransMessenger Transfection Reagent (Qiagen, Valencia, CA) following the manufacturer's protocol. Twenty-four hours after transfection, cells were incubated in the presence of LPS and IFN- γ for an additional 24 h. Cerebellar granule neurons were prepared from fetal mice (14-day gestation) derived from wild-type and nNOS knockout mice, and cultured in Basal Eagle Medium (BEM) containing 10% FBS, 25 mM KCl, and 2 mM L-glutamine. To activate NMDA receptor, eight ~10-day-old cultures were treated with Mg²⁺-free Earle's Balanced Salt Solution (EBSS) containing 300 μ M NMDA and 5 μ M glycine for 10 min. Cells were collected 24 h after NMDA exposure. For RNAi experiments, media for mouse cerebral granule neurons was changed to Neurobasal (Gibco BRL, Gaithersburg, MD) including B27 supplement 24 h before transfection at 5 days *in vitro* (DIV 5). Neurons were transfected at DIV 6 with 1 μ g of GFP construct and a total of 3 μ g of varied combinations of GAPDH, Siah1, Siah2, and control shRNA plasmids using Lipofectamine 2000. Neurons were fixed and prepared for staining with an anti-GFP antibody to enhance the signal.

Pulse-Chase Assay for Siah1 Protein. HEK293 cells transfected with HA-tagged Siah1 and Myc-tagged GAPDH or with HA-tagged Siah1 alone were

used for analysis. One day after transfection with the constructs, cells were starved in methionine-free DMEM and 1% dialyzed calf serum for 30 min, which was followed by a pulse phase with 1 mCi [^{35}S]methionine in methionine-free DMEM with 1% dialyzed calf serum for 1 h. After the pulse, the media was changed to methionine-free DMEM, 1% dialyzed calf serum, and 1 mM L-methionine. Cells were extracted with 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 0.3% SDS on ice. The supernatant produced after centrifugation at 14,000g for 20 min was used for further immunoprecipitation using anti-HA antibody for the analysis of Siah. The pellets of immunoprecipitation were analyzed on SDS-PAGE. The signals corresponding to Siah were quantified using a densitometer.

Immunofluorescent Cell Staining. Immunocytochemistry using confocal microscopy was performed as described (2).

Cytotoxicity. Cytotoxicity of HEK293 cells was monitored using an LDH assay kit (Sigma). Cell death in the LDH assay was calculated as follows. LDH in the media and within cells was measured independently. In parallel, we monitored the transfection efficiency by using the same amount of GFP construct. The LDH ratio of media supernatant to total cell extract was standardized by the transfection efficiency to obtain the percentage of dead cells. The neuroprotective effect afforded by transfected shRNA plasmid was monitored by co-expression of GFP. The molar ratio of shRNA plasmid to GFP plasmid was 3:1. Using fluorescent microscopy with a digital camera, we captured images of more than 20 fields per preparation, which were randomly chosen in a blind manner. GFP-positive neurons were tallied in each field and added together to determine the percentage of viable cells compared with control.

CAT Assay. N-CoR-mediated modulation of gene transcription was evaluated by CAT expression directed by a herpes simplex virus thymidine kinase (TK) promoter, which contained five Gal4 binding sites that were located 150 base pairs upstream of the TATA box, as described previously (27).

S-Nitrosylation Biotin Switch Assay. The assay was performed as described (20). In brief, cells were lysed and reduced cysteines were blocked with 4 mM methyl methanethiosulphonate (MMTS). Subsequently, S-nitrosylated cysteines were reduced with 1 mM ascorbate and biotinylated with 1 mM Biotin-HPDP (Pierce, Rockford, IL). The biotinylated proteins were pulled down with streptavidin agarose and analyzed by Western blotting.

NO_2 Concentration. The Griess reagent kit (Molecular Probes, Eugene, OR) was used following the manufacturer's protocol.

BIND Identifiers. Two BIND identifiers (www.bind.ca) are associated with this manuscript: 297416 and 297373.

NOTE: The Supplementary Information at the end of this chapter appears on the *Nature Cell Biology* Web site (<http://www.nature.com/naturecellbiology>).

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Supplementary Information

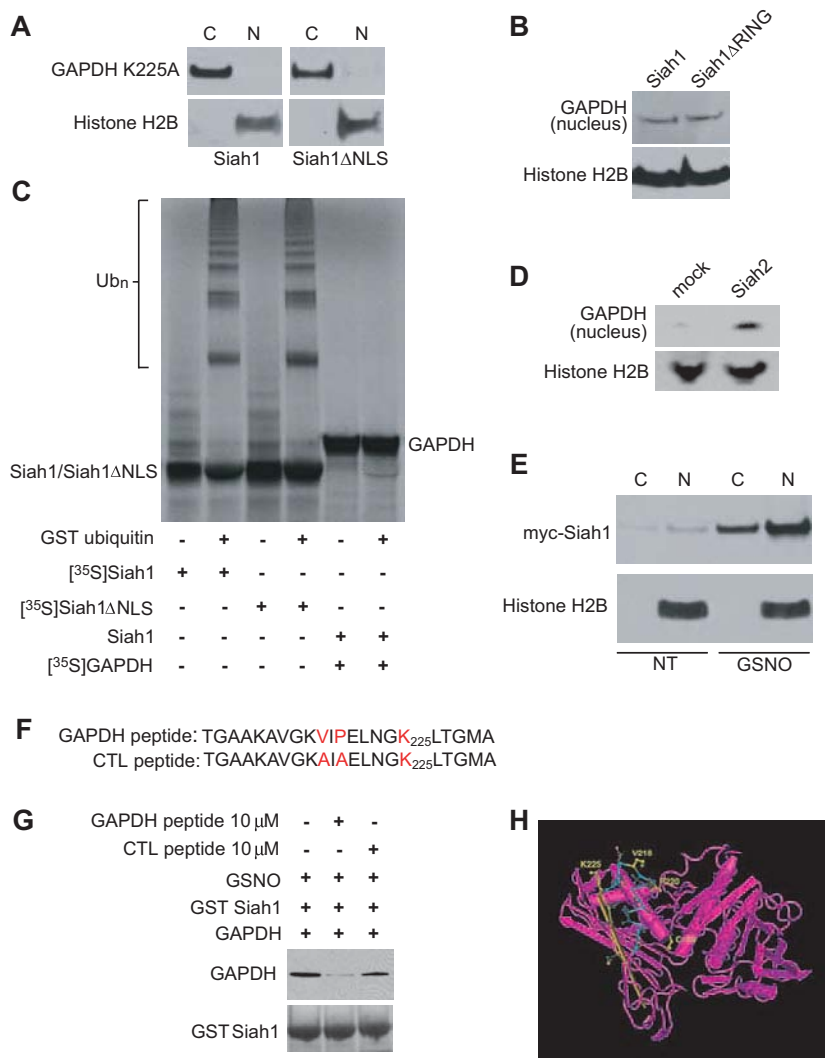


FIGURE 26–S1.

(A) Subcellular distribution of GAPDH K225A mutant was examined in cells transfected with Siah1 or Siah1ΔNLS. C: supernatant after 100,000g centrifugation, reflecting cytosol. N: purified nuclei obtained by sucrose gradient centrifugation. The result was replicated two times. (B) Siah1ΔRING mediates GAPDH’s translocation to the nucleus as well as wild-type Siah1. HEK293 cells were transfected with either Siah1 or Siah1ΔRING. Forty-eight hours after transfection, cells were harvested and crude nuclear fraction was purified. (C) *In vitro* ubiquitination assay of Siah1. *In vitro*

FIGURE 26–51. *(continued)*

transcribed and translated [^{35}S]Siah1, [^{35}S]Siah1 ΔNLS , or [^{35}S]GAPDH was used in the assay. Ub_n indicates the migration position of polyubiquitin conjugates. The result was replicated three times. (D) Siah2 enhances GAPDH's translocation to the nucleus. HEK293 cells were mock transfected or transfected with myc-Siah2 (human). Cells were harvested and the nuclear fraction obtained. The result was replicated two times. (E) Effect of an NO donor on Siah1 protein levels. myc-Siah1 was transfected into HEK293 cells. Transfected cells were either mock treated or 750 μM GSNO treated for 24 h. Augmentation of cytosolic Siah1 presumably reflects Siah1's capacity to shuttle between the cytosol and nucleus. The result was replicated two times. (F) Amino acid sequence of synthetic GAPDH peptide, which contains Siah binding motif, and control (CTL) peptide, which contains replaced amino acids in Siah binding motif (2). (G) GAPDH peptide inhibits the binding of GAPDH and Siah1. GAPDH was pretreated with 200 μM GSNO. GST-Siah1 (amino acid 109–282 of rat Siah open reading frame [cf. (A)] was added, and binding was assessed by GSH-agarose pull-down followed by Western blotting. The result was replicated three times. (H) Crystal structure of the rabbit GAPDH (3, 4). The Siah binding domain and C150 are depicted by a ball and stick model.

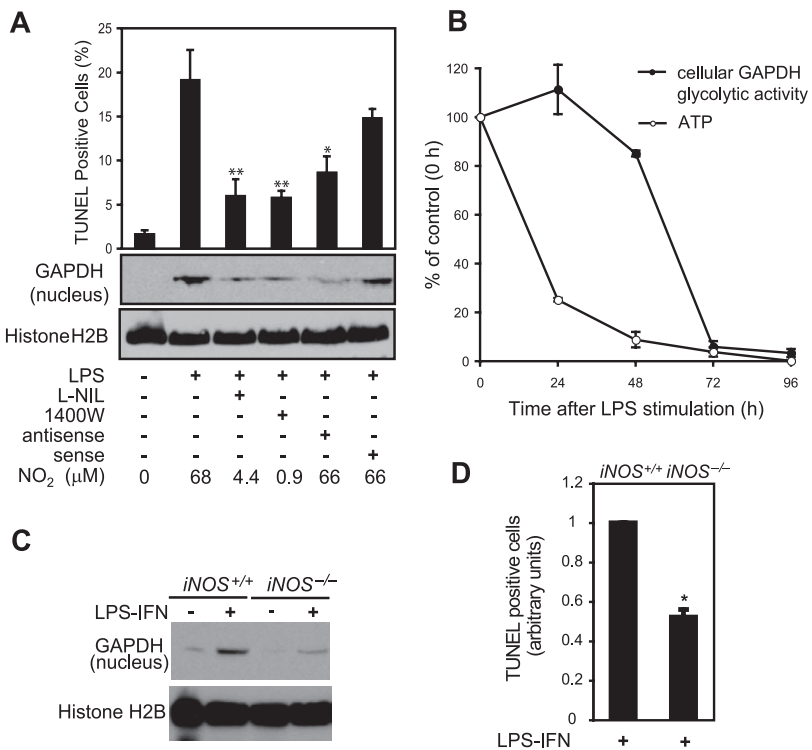


FIGURE 26–52.

(A) Depleting nuclear GAPDH protects macrophages from cell death caused by NO. RAW264.7 cells were activated with LPS for 72 h. iNOS inhibitors L-N⁶-(1-iminoethyl)lysine (L-NIL) and 1400W were added together with LPS. Antisense (5 μM) or sense (5 μM) oligonucleotides to GAPDH were added 1 h before stimulation with LPS. Cell death was assessed by TUNEL assay ($n=4-5\pm\text{SEM}$, $*P<0.05$ versus LPS; $**P<0.01$ versus LPS; $P=0.3$ for LPS+sense versus LPS only). Nuclear fractions were analyzed by Western blotting. Densitometry analysis reveals that 90 (10%) ($n=2$) of GAPDH translocates to the nuclear fraction 72 h after LPS treatment, consistent with decreased cellular GAPDH glycolytic activity as in (B). NO₂ concentration was measured by the Griess reagent ($n=4$). (B) Cellular GAPDH glycolytic activity and ATP in RAW264.7 cells after LPS stimulation. RAW264.7 cells were treated with LPS for the indicated period. GAPDH glycolytic assay: $n=3\pm\text{SEM}$. Cellular ATP: $n=2\pm\text{SEM}$. (C) Decreased translocation of GAPDH to the nucleus in peritoneal macrophages from iNOS knockout mice. Peritoneal macrophages were treated with LPS and IFN-γ for 24 h. The nuclear fractions were analyzed by Western blotting. The remaining nuclear translocation of GAPDH in iNOS knockout mice is presumably due to reactive oxygen species produced from NADPH oxidase, which is also activated by LPS-IFN-γ treatment. The experiment was replicated two times. (D) Endogenous NO is involved in cell death of peritoneal macrophages. Peritoneal macrophages were treated as in (C). Cell death was assessed by TUNEL assay ($n=3\pm\text{SEM}$, $*P<0.0005$).

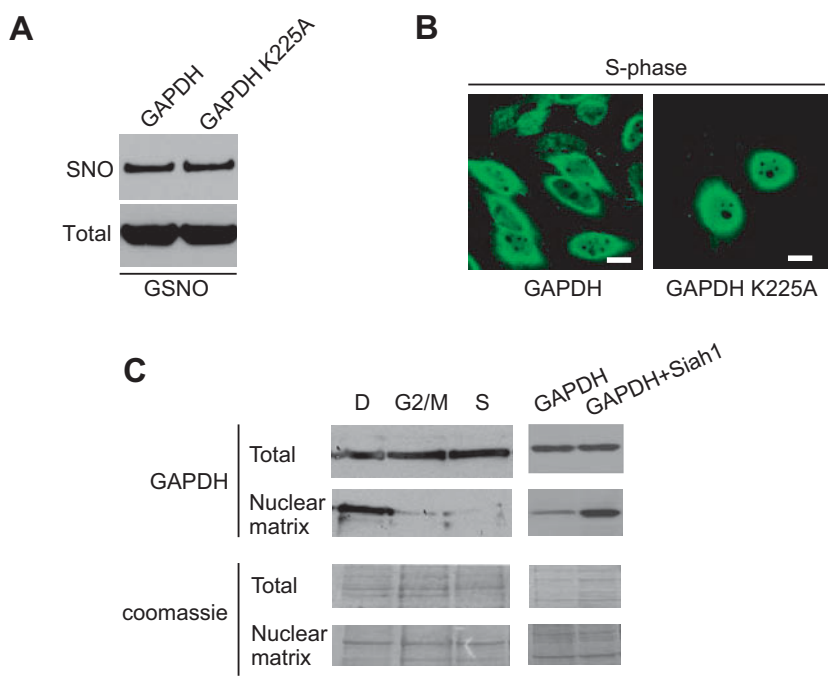


FIGURE 26-S3.

(A) S-Nitrosylation of GAPDH K225 mutant. HEK293 cells were transfected with either HA-GAPDH or HA-GAPDH K225A mutant. Cell lysates were treated with 50 μ M GSNO for 1 h at 37°C and subjected to the biotin switch assay. SNO: S-nitrosylated target proteins. The result was replicated two times. (B) GAPDH K225 mutant translocates to the nucleus during S-phase. HeLa cells, transfected with HA-GAPDH K225A, were synchronized in S-phase and stained with anti-HA antibody. Cells were examined under confocal microscope. As a control, endogenous GAPDH during S-phase was stained in parallel. The scale bar indicates 20 μ m. Representative pictures from two experiments are presented. (C) GAPDH in the nuclear matrix: Substantial levels of endogenous GAPDH were observed in the nuclear matrix during apoptotic cell death (16 h after 100 μ M etoposide treatment) in HeLa cells (D: Death). Nuclear GAPDH in HeLa cells synchronized in the S-phase is soluble and does not occur in the nuclear matrix (G₂/M, S). GAPDH overexpressed with Siah1 in HEK293 cells is concentrated in the nuclear matrix (GAPDH, GAPDH+Siah1). The result was replicated two times.

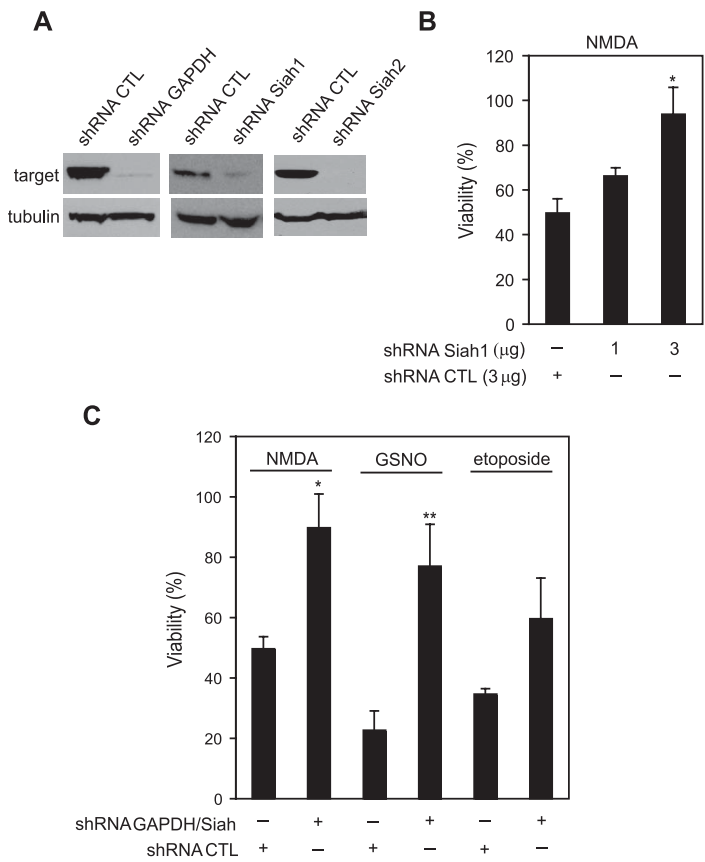


FIGURE 26–54.

(A) Target protein depletion by shRNA examined by Western blotting. Expression constructs to a target molecule (GAPDH, Siah1, Siah2) with myc-tag were transfected into HEK293 cells together with a plasmid construct expressing shRNA to each target or control shRNA (shRNA CTL). Two days after transfection, total cell extracts were analyzed on SDS-PAGE followed by Western blotting with an anti-myc antibody. The experiment was replicated three times. (B) Dose dependent protection of shRNA-Siah1 against NMDA neuronal toxicity. Neurons were treated with NMDA as in Figure 26–7C, except transfection of either 3 μg shRNA CTL ($n=5\pm\text{SEM}$), 1 μg shRNA Siah1 ($n=2\pm\text{SEM}$), or 3 μg shRNA Siah1 ($n=4\pm\text{SEM}$, $*P<0.01$). Viability was assessed by GFP staining. (C) Comparison of shRNA to GAPDH and Siah in preventing cell death elicited by NMDA, GSNO, or etoposide. Primary cerebellar granule neurons were transfected with plasmid expressing shRNA to CTL or combination of GAPDH, Siah1, and Siah2 (shRNA Siah: shRNA Siah1+shRNA Siah2). Transfected cells were treated either with NMDA ($n=5\pm\text{SEM}$, $*P<0.01$), same as in Figure 26–7C, treated with 200 μM GSNO for 48 h ($n=3\pm\text{SEM}$, $*P<0.05$), or 100 μM etoposide for 24 h ($n=2\pm\text{SEM}$). Viability was assessed by GFP staining.

Supplementary Methods

Reagent. GAPDH peptide [TAT domain (YGRKKRRQRRR)-TGAAKAVG KAVGKVIPELNGKLTGMAK-5FAM] and a control peptide [TAT domain (YGRKKRRQRRR)-TGAAKAVGKAVGKAIAELNGKLTGMAK-5FAM] were produced by GenScript Corporation. Antisense and sense phosphorothioate oligonucleotides to mouse GAPDH were produced in the DNA synthesis facility at Johns Hopkins University. The oligonucleotide sequences for mouse GAPDH were 5'-GACCTTCACCATTTGTCTA-3' for antisense, and 5'-TAGACAAGATGGTGAAGGTC-3' for sense. L-NIL was purchased from Alexis.

TUNEL Assay. Cell death of RAW264.7 cells was assayed 72 h after LPS (1 μ g/mL) exposure. Antisense (5 μ M) or sense (5 μ M) oligonucleotides to GAPDH were added to the media 1 h before the LPS exposure. L-NIL (100 μ M) or 1400W (100 μ M) was added together with LPS. Total and dead cells were determined by counting nuclei stained with 100 ng/mL DAPI and TUNEL, respectively. Cells were fixed in 4% paraformaldehyde and then stained using the *In Situ* Cell Death Detection Kit (Roche) following protocols provided by the manufacturer. The cells were examined under a fluorescence microscope (Zeiss). Cell death was determined as the ratio of dead to total cells.

Cell Synchronization. The well-established protocol using thymidine was used as described (1). In brief, HeLa cells were treated with 2 mM thymidine for 16 h to initiate an S-phase block. Cells were released from the initial block by washing off thymidine and incubating in medium containing 24 mM deoxycytidine for 9 h, allowing progression through the cell cycle. A second block with thymidine was for 16 h to synchronize cells at the G1/S boundary.

***In vitro* Ubiquitination Assay.** Siah1, Siah1 Δ NLS, and GAPDH were labeled with [35 S]methionine by *in vitro* translation by using TnT kits (Promega). Two μ L of *in vitro* translated sample were mixed with 100 ng E1 (BostonBiochem), 200 ng UbcH5b (BostonBiochem), and 10 μ g GST-Ubiquitin (BostonBiochem) in a reaction mixture (50 mM Tris pH 7.4, 2 mM ATP-Mg $^{2+}$, 2 mM DTT, 5 mM MgCl $_2$). The sample was incubated for 60 min at 30°C, resolved by SDS-PAGE, and analyzed by autoradiograph. For examining ubiquitination of GAPDH by Siah1, Siah1 was *in vitro* translated with cold methionine and 4 μ L of the sample were used for ubiquitination assay.

Enzymatic Assay for GAPDH. Cells were harvested and lysed in HEN buffer (250 mM HEPES pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine). Crude lysates were cleared of insoluble debris by centrifugation at 1,000g. The assay mixture contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium arsenite, 2 mM NAD $^{+}$, 500 μ M DTT, and 10 μ g of cell lysates. After incubating in a spectrophotometer at 37°C for 5 min to achieve temperature equilibrium, the reaction was initiated with the addition of glyceraldehyde-3-phosphate to 2 mM. A $_{340}$ from 0 to 10 min was recorded.

Measurement of ATP. ATP concentration was measured by chemiluminescence using Bioluminescent Somatic Cell Assay Kit (Sigma) following the manufacturer's protocol.

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Part X

WHAT MAKES FOR CREATIVE DISCOVERY IN SCIENCE?

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COMMENTARY

What Creates Creative Science and Scientists?

Nancy C. Andreasen

The title of this section formulates one of the “big questions” in science. Unfortunately, it is one that people rarely ask, and so we have no good empirical database from which to answer it. There are very few empirical data-based studies of creativity, and most have emphasized the arts rather than the sciences. A few good studies of creative scientists were done during the 1960s, but these relied heavily on anecdotal interviews and psychological tests that are relatively rudimentary and unsophisticated, at least when examined with the retrospective eye of twenty-first-century research.

After finishing my own empirical studies of creative writers recruited from the renowned Iowa Writers’ Workshop in the 1980s, I vowed that I would complement that work with the study of other forms of creativity, including creativity in science. One reason that we have so few studies of creativity is that such studies are difficult to undertake. Although the results of research on creativity should have far-reaching implications for education, scientific policy, and even currently popular initiatives such as national defense, there is no obvious funding agency willing to support research of this type. That problem, coupled with my own consuming interests in imaging and schizophrenia, caused me to keep my vow on hold until very recently. A small grant from a generous private donor who wanted to fund creativity research catalyzed a decision to begin my new project and to design a study of creativity that would include creative scientists.

Although the project has not yet begun, I thought it might be helpful to contact one or two people to check on their willingness to take part. Because

the study will include functional magnetic resonance imaging, and there is a better chance of getting robust activations from younger people, my first contact was a relatively young Nobel Laureate (whom I'll leave unnamed). He responded that he didn't think he should participate because he didn't consider himself to be very creative, in comparison with many other scientists, despite his Nobel Prize. He said he was primarily methodical and persistent rather than creative. So I asked him to identify a few scientists whom he considers to be really creative. Guess who was near the top of his list—the person whose work this book anthologizes, our very own Sol Snyder. (Sol didn't know this story until he received this introduction to his article on the “audacity principle” [1; reprinted as Chapter 27 in this volume]. He was on my list anyway, but he didn't yet know that he might be stuck making a trip to Iowa City and sticking his head in an MR scanner.)

As this anecdote indicates, it is very appropriate that Sol and I put our heads together, through his article and this introduction, and consider what we do know about creativity in general and creativity in science in particular.

One thing we know is that creativity and intelligence are different mental traits. They may coexist in some people (such as Sol), but this is not necessarily the case. The “highly intelligent” person is usually defined as someone with an IQ in the range of 140 or greater. The mean IQ in groups of people selected because they are highly creative is considerably lower. The average IQ of the writers in my Iowa Writers' Workshop study was 120. This is usually the average in most group studies of creative people.

So what are the factors that make for creativity, if it is not a consequence of having a high IQ? Well, Sol uses the term *audacity*, and that is not a bad choice. It is a reasonable proxy for other terms that I have used when I have written about the creative person and the creative process. Creative people tend to be more willing to take risks than other people. They develop ideas that are ahead of their time, and they frequently spend a decade or so confronting skepticism, doubt, and rejection. They have a capacity to believe in their ideas and to persist in pursuing them without accolades or rewards. They are sometimes seen as rebels because they refuse to accept received ideas and traditional teachings. They see a new reality that is not visible to others with more mundane minds. As Sol says, they are courageous. They love simplicity. And they love to make connections where others cannot perceive them. They don't need to spend years in libraries “reviewing the literature.” They know that the best ideas arise from the insights created in their own minds.

If this kind of cognitive style is the hallmark of the creative scientist, what are the implications for education and science policy? That we should be doing things very differently than we currently are.

Every good scientist who must be funded by the current peer review system knows that he or she must play a game that is antithetical to the nature

of good science. The game begins with an introduction containing a weighty and extensive “review of the literature.” Then there is the section on hypotheses. The hypotheses can’t be really original and interesting, because the reviewers will dismiss them as implausible. Endogenous opioid receptors? Impossible! Gases acting as neurotransmitters in the brain? You have to be kidding! Neurotransmitters that are not released from synaptic vesicles? Ridiculous! And then after those very mundane and conventional hypotheses, one must lay out a five-year plan of work—as if new techniques and technologies will not arise or be developed that might replace current ones in the next year or two. And then the statistics and power analyses—these must be described in picky and pedantic detail, as if flashes of insight that can be quickly verified by (as Sol describes) finding *the right simple experiment* have no place in the world of science. Our current methods for identifying good science and funding it use principles that are antithetical to actually doing good science. They reward caution, conservatism, and conventionality. I keep hoping that those who set science policy will begin to understand the value and virtue of audacity and find ways to recognize and reward it. And I hope some of those policy makers will read this book, Sol’s article on the audacity principle, and this introduction to it. And then I hope that they will have the audacity to change things so that creative science can grow more freely.

Another thing that we know about creativity, also highlighted in Sol’s “audacity principle” article, is that mentors or patrons can play an important role. If an artist or scientist is taking a new path that is likely to lead to being rebuffed or doubted, he or she needs encouragement in order to persist. Mentors and patrons play an important role in providing psychological support to a young person with creative talent. They frequently also provide material support. For Michelangelo, Lorenzo de’ Medici provided marble and a studio where the young genius could learn the lost art of sculpting in stone. Mentors for young scientists provide laboratory facilities in which ideas can be tested, and tested again, until the right technique is found to answer the questions being asked. And perhaps most importantly, good mentors are good role models. The best ones, like Julie Axelrod, demonstrate vividly and teach avidly that people with original ideas can ultimately succeed. A few years ago the National Institutes of Health discontinued its Career Scientist program, on the grounds that more money must be spent on young scientists. Although the goal was salutary, the consequence is that senior mentors now have less time to mentor, since they must scramble to support their salaries directly from research grants—competing with the very same young investigators whom the policy was supposed to benefit. Most of us who love to teach do it anyway, without incentives and often with sacrifices. But perhaps NIH should consider creating a small program to reward mentors, as an important public statement.

Yet another thing that characterizes the creative mind, highlighted in “The Audacity Principle,” is its difficulty in seeing boundaries between ideas, disciplines, or even just objects, that others believe to be present with a high level of certainty. A creative mind may play with ideas picked up from computer science and apply them to models of brain activity. Ideas from physics, such as chaos theory, may lead to the recognition that the human brain is a classic self-organizing system. Many of our greatest creators had no idea that “art” and “science” should be considered to be very different and disparate disciplines. Leonardo composed music, dissected bodies and produced beautiful anatomical drawings, designed military fortifications and equipment (including helicopters and tanks), cast statues in bronze, and did architectural designs. He also painted a bit.

This observation also has implications for education and public policy. If we want to create environments that will nurture creativity in children, adolescents, and young adults, those environments should not be filled with silos where the arts are isolated from the sciences and the subdivisions within each create further isolations. Tracking and specializing early may benefit some, but encouraging breadth and diversity is likely to benefit many. We need to revive the concept of the polymath—the person who knows many different fields and is good at lots of them. A richly varied educational background provides fertile soil for creativity. As an English professor turned neuroscientist, I have many anecdotes to support this generalization, but I’ll save them for my own article on the value of audacity.

Thanks to you, Sol, for this summary of your views on the audacity principle and on how it has permitted you to achieve a remarkable career. It provides refreshing insights on how creative science works, based on the experiences of someone who has been doing it for many decades.

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CHAPTER 27

The Audacity Principle in Science

Solomon H. Snyder

DEDICATED TO JULIUS AXELROD
a humble, gentle scientist who nonetheless
epitomized the Audacity Principle in Science.
He died 29 December 2004.

My mentor, Julius Axelrod, often commented, “Ninety-nine percent of the discoveries are made by one percent of the scientists.” This may sound like an exaggeration. However, a brief examination of the major advances in any branch of science reveals the truth of this dictum. Axelrod himself is a prime example. In the field of molecular pharmacology, many of the key advances are attributable to his own efforts. He elucidated the metabolism of the major psychoactive drugs, in the process laying the groundwork for the emergence of acetaminophen (Tylenol) as a major analgesic and then uncovering the family of drug-metabolizing enzymes, now known as the P450 enzymes. He accomplished most of this while working as a laboratory technician without a Ph.D. Following receipt of his doctorate at age forty-two, Axelrod proceeded to revolutionize neurotransmitter research. Consider the catecholamine neurotransmitters such as norepinephrine and dopamine. After norepinephrine was established as the neurotransmitter of sympathetic

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nerves in the late 1940s, advances were relatively modest. The enzymatic processes leading to its biosynthesis from the dietary amino acid tyrosine were gradually elucidated by multiple investigators over a period of several decades. Classical pharmacologic studies comparing the effects of different drugs on sympathetic neurotransmission had led to an appreciation that there were at least two subtypes of receptors for norepinephrine, designated alpha and beta, which subsequently led to important new drugs. Then, in roughly half a decade, Axelrod had a series of insights that drastically altered our thinking about norepinephrine and, indeed, all neurotransmitters. In 1957 he discovered catechol-*O*-methyltransferase, a key enzyme in metabolizing the catecholamines. This led him to question the prevailing assumption that the only other known enzyme that metabolizes catecholamines, monoamine oxidase, accounts for inactivation of norepinephrine after it is released by sympathetic nerves. Inactivating neurotransmitters is of crucial importance, for it serves to remove them from the vicinity of receptors on adjacent neurons so that successive nerve impulses will be effective. In the early 1960s the only neurotransmitter known besides norepinephrine was acetylcholine, discovered in the late 1920s. It was well established that the actions of acetylcholine are terminated by enzymatic degradation via an enzyme called acetylcholinesterase. Drugs that inhibit this enzyme potentiate the actions of acetylcholine at synapses, sites where nerves communicate with each other. Such acetylcholinesterase inhibitors provide important therapy for diseases such as myasthenia gravis that are characterized by muscle weakness because of deficient neurotransmission at acetylcholine synapses.

Because of the well-established role of acetylcholinesterase in inactivating acetylcholine, it was accepted wisdom that enzymes would inactivate norepinephrine at its synapses. But no one had directly evaluated whether monoamine oxidase was in fact responsible for norepinephrine inactivation. To compare the roles of catechol-*O*-methyltransferase and acetylcholinesterase, Axelrod utilized drugs that inhibit the two enzymes and was surprised to find that neither enzyme could explain synaptic inactivation. About this time, radioactive forms of norepinephrine became available. Instead of pursuing convoluted biochemical experiments with the radiolabeled molecule, Axelrod simply injected it into rats. He was amazed to find that organs enriched in sympathetic nerves, such as the heart, enormously concentrated the radioactive norepinephrine. When sympathetic nerves were severed, these organs no longer took up the neurotransmitter, indicating that it was the sympathetic nerve endings that had been concentrating norepinephrine. Based on his experimental findings, Axelrod formulated a simple model to explain neurotransmitter inactivation. He proposed that uptake of the norepinephrine into the nerve endings that had released it me-

diates synaptic inactivation. His theory was rapidly proven right by experiments showing that cutting the nerve endings to eliminate the uptake process markedly potentiated neurotransmission. He wondered whether drugs that could mimic the effects of sympathetic nerve firing might act by inhibiting this uptake process, thereby potentiating actions of norepinephrine. Utilizing radiolabeled norepinephrine, he soon showed that agents such as cocaine, amphetamines, and, most important, the major antidepressants, exert their effects by inhibiting the uptake process. Soon, other scientists showed that uptake mechanisms account for inactivation of virtually all the major neurotransmitters, with enzymatic degradation, as with acetylcholine, being the exception rather than the rule.

For these contributions, Axelrod shared the Nobel Prize in Physiology or Medicine in 1970 with Ulf von Euler, who established norepinephrine as a transmitter, and Bernard Katz, who showed that neurotransmitters are stored in and released from synaptic vesicles.

What do we learn from the above anecdote? First, in this field one scientist with a tiny laboratory comprising no more than three or four postdoctoral fellows could make many if not most of the key breakthroughs in a large field of research. Second, we learn to wonder what differentiates individuals such as Axelrod from others in the field. This leads us to the focus of this essay: what makes for greatness in scientific research?

Clearly Axelrod manifested an abundance of creative insights. He conceptualized principles never previously enunciated. The notion that neurotransmitters were inactivated by being taken back into the nerve that had released them initially met with ridicule. Axelrod saw through dogma as gerymandered as the Ptolemaic planetary system and, like Galileo, provided radical simplification. But creativity isn't enough. Whenever a major new discovery is published, dozens of scientists exclaim, "I thought of that a long time ago but just didn't do the right experiment." Original ideas are only a part of the story. A special sort of energy is required to overcome the fear or inertia that hinders scientists from essaying risky, unprecedented experiments. Of course, devising the optimal experiment is crucial, and all experimental breakthroughs involve simplification. One could conceptualize intricate, year-long approaches to experiments to explore neurotransmitter uptake. Axelrod simply injected radiolabeled norepinephrine into rats and came up with the "answer" in a day or two. Experimental ingenuity, a shrewdness in experimental design, and "good hands" all play a role in coming up with the "right" experiment. But I have known many talented experimentalists who never make major advances.

Much has been written about the nature of scientific discovery. The rigor of scientific hypothesis formulation and testing, as well as critical thinking to rule out artefactual explanations of data, is often highlighted. My personal

experience over three to four decades tells me that the real breakthroughs don't happen this way. The greatest scientists tend to resemble artists in certain ways, but with notable differences. Artists see the world differently than the rest of us. They find wonder in the seemingly mundane. They detect commonalities among objects that most viewers see as notably disparate. Similarly, the finest researchers view with puzzlement established principles that are taken for granted by the scientific community. In particular, they become irritated by overly convoluted explanatory principles. They seek simplicity, whereupon novel concepts emerge. The ability to divine unifying notions out of a morass of data seems critical.

Equally important is the intellectual and often emotional courage to enunciate such simplifications. Courage is requisite for many reasons. Challenging established authority is always risky. The challenge is even more complex in science because, when first presented, a new unifying concept can rarely account for all the available data. One must be willing to proffer and defend a novel hypothesis in the face of some contradictions, based simply on the argument that the virtues of a new model, compared with older formulations, exceed its drawbacks. Often history bears out the validity of the new paradigm, but sometimes the innovative notion proves false; hence the risk. Positing something new even in the presence of discrepancies is justified, for often such discrepancies fade away as new data emerge. The late Francis Crick was a lover of elegant, simple, and revolutionary hypotheses. He believed strongly in the beauty and simplicity of nature and thus favored simple explanatory models. In an informal group in which he and I participated years ago, he put it roughly this way, "If the theory has a beautiful feel and makes good sense despite some ugly data which don't agree, perhaps the data are wrong!"

All these factors seem to be relevant. Originality and simplicity are certainly crucial elements. Even more important are the intellectual fearlessness and emotional drive to put it all together, step forward, do the right experiment, promulgate it to the world, defend the new insights, and go forward to further innovation. A simple designation for this behavioral pattern might be the Audacity Principle. Audacious behavior is usually regarded as a form of hubris or chutzpah, an off-putting and overly aggressive behavior that we don't usually link to creativity. Here I use the term to focus on the intellectual qualities of audacity that enable individuals of talent to implement their own native ability. In other words, scientific originality may not be so rare a commodity as is the capacity to appreciate the importance of one's own ideas and to put them into practice.

Lineage

One way to seek the qualities that make for scientific discovery is to examine what is conveyed in the mentor-apprentice relationship. The eminent sociologist Harriet Zuckerman has provided compelling evidence for the importance of scientific lineage. She compared the careers of Nobel laureates with others, matched for closely similar educational background, intelligence, association with distinguished universities, and other factors, who were very good but not “Nobel quality” (1). The single factor that most clearly differentiated Nobel laureates from outstanding but lesser scientists was training with another Nobel laureate. Zuckerman distinguished the mentor-student personal relationship from the political contacts offered by a prominent scientist, as most future Nobelists trained with their mentors long before the latter had attained scientific eminence, far less a Nobel Prize.

Individual testimonies convey the role of mentorship. The biochemist Hans Krebs commented, “If I ask myself how it came about that one day I found myself in Stockholm, I have not the slightest doubt that I owe this good fortune to the circumstance that I had an outstanding teacher” (1, p. 124). What do mentors convey? The best teaching is done by example. There is an important emotional element whereby the mentor enables the student to feel self-confident enough to pour forth his/her original ideas. This mode of teaching is similar to what the best psychotherapists do with their patients, or parents with children. For the psychologist Carl Rogers the essence of psychotherapy was conveying to the patient an attitude that he designated “unconditional positive regard.” All good parents know that their children are never “bad children” but are instead good children who occasionally do bad things. Reinforcing the “good” and not punishing but merely disregarding the “bad” enhances self-esteem. I remember presenting my mentor, Julie Axelrod, with a bundle of experimental results that seemed like a total failure. Julie looked through the results and commented, “Sol, why are you so glum? Some of the findings aren’t so great, but, look! I see kernels of exciting ideas we can explore further.” When one considers that easily nine out of every ten experiments fail, such pep talks are invaluable.

A number of Zuckerman’s findings can be subsumed under the Audacity Principle, e.g., being sufficiently courageous to ask “important” questions. One might think that anybody’s grandmother can tell you what is important: “Go find the cure to cancer!” In reality, choices are far more subtle. Many molecular pathways lead to cancer, so it is hard to know which is “more important.” Moreover, scientists like everyone else are subject to a powerful herd instinct, jumping on the current, fashionable area, one for which experimental tools are usually readily available so that obtaining publishable data is relatively risk-free. Thus, asking the important question requires a combination

of creative insight and audacity. The great biochemist Otto Warburg, who studied with Emil Fischer, the second scientist to be awarded the Nobel Prize in chemistry, commented, "I learned (from Fischer) that a scientist must have the courage to attack the great unsolved problems of his time...without much critical hesitation" (1, p. 128). This courage to "go for it" without obsessive procrastination and excessive self-criticism is a hallmark of the greatest scientists. As Zuckerman said in summarizing her observations, "Among the elite scientists, the prime criteria of scientific taste are a sense for the 'important problem' and an appreciation of stylish solutions."

The writer Robert Kanigel, who has chronicled the careers of leading scientists, distills their lessons: "Just go with your hunch, your scientific intuition and isolate that single, elegant, pointed experiment that will tell you in a flash whether you are on the right track." He also noted, "*Just do it*, don't spend all year in the library getting ready to do it. Don't wait until you've gotten all the boring little preparatory experiments out of the way. Don't worry about scientific controls except the most rudimentary" (2, p. 236).

There is a knack to identifying important questions, which good mentors convey. Robert Kanigel described it this way: "Don't bother with the routine scientific problems.... Leave those to others. Don't bother, either, with big, fundamental problems which are simply not approachable with available techniques and knowledge; why beat your head against the wall? Half the battle is asking the right question at the right time" (2, p. 235). Axelrod said similar things: "One of the most important qualities in doing research, I found, was to ask the right questions at the right time. I learned that it takes the same effort to work on an important problem as on a pedestrian or trivial one. When opportunities came, I made the right choices" (3).

Let me illustrate how original ideas, simplification, experimental ingenuity, and willingness to take risks manifest the Audacity Principle in my own work. I do not claim to possess these qualities in greater abundance than other scientists, but cite my own examples simply because I know them best.

Receptors

Much of my professional life has dealt with studies of neurotransmitters. In 1970 scientists knew a great deal about their biochemistry, how they are formed, stored, released, and inactivated by reuptake. Virtually nothing was known about the most critical actions of neurotransmitters, namely, their ability to act in a lock-and-key fashion upon receptors on adjacent target cells. Then, within a span of a few months, several research groups reported success in identifying a receptor for acetylcholine, the first-discovered and best-characterized transmitter. The investigators took advantage of a re-

markable structure, the electric organ of the electric eel. The electric organ is extraordinarily enriched in these receptors, so much so that they generate sometimes lethal shocks to the eel's prey. The researchers also took advantage of an extraordinarily potent snake toxin, alpha-bungarotoxin, which binds with high affinity to the receptors. The unique properties of this system suggested that it would be impossible ever to identify biochemically the receptors for neurotransmitters in the brain. Thus, the acetylcholine receptors constitute 20 percent by weight of the electric organ of some eels, whereas one could calculate that most neurotransmitter receptors in the mammalian brain would be only about one millionth by weight. Moreover, there were no magical toxins available for most neurotransmitter receptors.

I was aware of the importance of identifying neurotransmitter receptors, but chose instead to address receptors for opiate drugs, largely because research in our laboratory was funded by the drug abuse division of the National Institutes of Health. At that time nothing was known about the nature of receptors for drugs such as opiates, other than their ability to bind the drugs. One could obtain radiolabeled drugs and monitor their binding to brain membranes, but armchair calculations would tell you that this should be impossible. Based on the known potencies of opiate drugs in intact mammals, the distinguished pharmacologist Vincent Dole had estimated the quantity of presumed opiate receptors in the brain and had come up with a number that was about the same as the expected density of most neurotransmitter receptors, namely, about one millionth by weight. Opiate drugs possess positively charged nitrogen atoms that might bind nonspecifically to negatively charged tissue constituents. The drugs also possess benzene rings that would bind nonspecifically to lipid-containing constituents. Such nonspecific binding would surely vastly exceed the number of specific receptor binding sites.

To overcome such challenges, my students and I reasoned that the biologically relevant opiate receptors should have a far higher affinity for opiate drugs than the nonspecific binding sites. One could take advantage of this by utilizing opiates of high specific radioactivity, i.e., with a great deal of radioactive label per molecule, so that one could employ very low concentrations of the radiolabeled drug that would bind more selectively to specific receptors than to nonspecific sites. Moreover, drug molecules that were bound with high affinity to receptors would wash away more slowly than nonspecifically bound drug molecules. Thus, one would wash the brain membranes extensively to remove nonspecific binding while preserving true receptor interactions. Of course, such washing would have to be done very rapidly, lest the radiolabeled opiate also wash off the receptors. This strategy enabled us to identify opiate receptors (4).

To implement this receptor-binding strategy on a large scale, I borrowed from my colleague Pedro Cuatrecasas a vacuum filtration manifold he de-

vised for his pioneering studies of insulin receptors. With this apparatus we were able to process hundreds of samples in an hour, enabling us to do far more than simply establish that the brain possesses pharmacologically relevant opiate receptors. We could examine large numbers of drugs rapidly. This permitted us to explore the actions of numerous drugs. For instance, we were able to explain key pharmacologic actions of codeine and heroin. Codeine, which acts gradually to relieve pain and cough, didn't bind to opiate receptors at all. Codeine differs from morphine solely by the addition of a methyl group that covers up a hydroxyl structure that is critical for receptor interactions. After ingestion, the methyl group of codeine is removed in the liver, generating morphine that then enters the brain. Hence, codeine is nothing but a pro-drug for morphine. The fact that it must be metabolized before it can act explains its gradual onset of action. Any drug that enters the brain gradually is much less likely to produce a "high." This explains the greater safety of codeine as compared to morphine in terms of abuse. Heroin also failed to bind to the opiate receptor. Interestingly, the pharmacology of heroin is the opposite of that of codeine. After intravenous injection heroin enters the brain very rapidly, causing the "rush" of euphoria that underlies its massive addictive potential. Heroin doesn't bind to opiate receptors, because it possesses an acetyl group that overlies the critical hydroxyl of morphine. In contrast to the stability of the methyl group in codeine, which requires removal by enzymes of the liver, the acetyl group of heroin is spontaneously liberated when heroin enters the brain. Thus, heroin, like codeine, is a pro-drug of morphine, but it delivers the morphine far more rapidly.

Since we could measure large numbers of samples rapidly, we were able to dissect monkey brains into tiny regions, and thus discovered that the relative densities of opiate receptors in various areas of the brain could fully account for major pharmacologic actions of the drug (5). For instance, opiate receptors were extremely concentrated in the lateral region of the thalamus, an area where sensory information is processed. Moreover, the lateral thalamus processes information about chronic, aching pain, the sort that is relieved by opiates, in contrast to the medial thalamus, which deals with brief, sharp pin-prick types of pain that don't respond to the drugs. Opiates are well known to constrict the pupils of the eye, enabling police to identify an addict at a glance. Opiate receptors were highly enriched in certain nuclei of the brain stem that regulate pupillary diameter.

These are but a few of the myriad insights into opiate actions that derived from the ability to measure them in simple test-tube systems. What does this reveal about behaviors that make for scientific success? First of all, most scientists would never have embarked on the search in the first place. Their carefully considered armchair reasoning would have told them that the task was fruitless. Moreover, much preplanning was required. To seek opiate re-

ceptors, we had to obtain, at great expense, custom-prepared radiolabeled forms of the opiate antagonist naloxone. Mastering the vacuum filter manifold took time. Nonetheless, with the “uncritical” foolhardiness of youthful optimists, my students and I obtained the appropriate radiolabeled drug and moved forward. Suspending one’s critical faculties at key times and moving forward with seeming impetuosity have contributed to many major advances in science. In almost all instances known to me, the most successful scientists have addressed the riskiest projects and have thus encountered more failure than success. But, as in venture capital investing, a few major successes more than compensate for large numbers of failures.

Another component of success in science is what detractors refer to as opportunistic exploitation of discoveries. By this, they mean applying the fruits of one discovery to other, related “easy” findings. Thus, when Axelrod discovered catechol-O-methyltransferase, the enzyme that adds a methyl group to norepinephrine, he reasoned that there must be many other methylating enzymes. He thus went about seeking and finding the enzyme that generates the pineal gland hormone melatonin, the enzyme that forms the adrenal gland hormone adrenaline/epinephrine, and many others. His scientific critics described this approach as “going for the low-hanging fruit.” On the contrary, I regard Axelrod’s approach as a way to uncover major new insights with minimal effort and I have never shirked from such scientific opportunism. Since the properties of the opiate receptor were very much like what one would expect of a neurotransmitter receptor, we applied receptor technology, with various modifications, to an assault on all the major neurotransmitter receptors in the brain and, by the mid-1970s, had successfully labeled most of them. For instance, in the mid-1970s there was great interest in the neurotransmitter dopamine. Degeneration of dopamine neurons in the brain accounts for the principal symptoms of Parkinson’s disease. L-Dopa, the amino acid precursor of dopamine, was first employed therapeutically in the late 1960s as a drug that replaces the missing dopamine and alleviates most symptoms. There were also hints, from the work of the Swedish pharmacologist Arvid Carlsson, that the antischizophrenic actions of the class of drugs called the neuroleptics, exemplified by agents such as chlorpromazine and haloperidol, might act by blocking dopamine receptors. Inability to measure dopamine receptors directly precluded an investigation of this hypothesis. Utilizing radiolabeled dopamine and haloperidol, we identified dopamine receptors by the same binding technology that worked with opiate receptors (6), findings obtained independently by Philip Seeman in Toronto (7). We showed that the relative potencies of neuroleptic drugs in blocking dopamine receptors paralleled closely their potencies in relieving schizophrenic symptoms, establishing the mechanism of the antipsychotic actions of the drug. The “opportunism” involved in transferring opiate receptor

technology to a variety of other receptors seems to be another manifestation of the Audacity Principle.

Man was not born with morphine in him. The fact that opiate receptors resembled neurotransmitter receptors suggested that there must exist opiate-like neurotransmitters. We were able to demonstrate that such substances exist (8), as did the Swedish pharmacologist Lars Terenius (9) and Scottish investigators John Hughes and Hans Kosterlitz (10). The Scottish group and our group developed approaches to isolating the substances and obtaining their chemical structure. In a brilliant opus the Hughes-Kosterlitz team reported in December 1975 the chemical structure of the two enkephalins, the first of the endorphins, neurotransmitters that mimic the actions of morphine and are major regulators of pain and emotional perception (11), findings we obtained as well soon thereafter (12).

Conceptualizing that endogenous morphine-like substances should exist was important and reflected an appreciation of “the big question,” but many scientists had come to similar conclusions. One needed the gumption to “do something.” Moreover, divining how to transform the big question into small, soluble parcels was particularly critical.

Our lab and the Kosterlitz group designed very different ways of approaching the problem. As experts on opiate receptor binding, we looked in brain extracts for materials that would compete with radioactive opiates for binding to the receptors. A master of classical pharmacology, Kosterlitz took advantage of the known constipating actions of morphine, monitoring in a simple organ bath the ability of brain extracts to mimic the capacity of morphine to inhibit electrically induced contractions of the gut. With a simple system to measure the morphine-like activity of brain extracts, both groups could purify the enkephalins.

When we embarked on the search for the enkephalins, we already knew that Hughes and Kosterlitz had identified such substances and had made considerable progress in purifying them. Many scientists would have shied away from the competition. They would not even have conceptualized a way of addressing the problem, for they would assume that others had already covered the ground. Our willingness to accept the challenge again reflects the Audacity Principle.

Gases as Neurotransmitters

In the late 1980s I read a paper in *Nature* by Salvador Moncada (13) describing his experiments establishing conclusively that nitric oxide (NO) was the physiologic molecule that accounts for endothelial-derived relaxing factor activity. I was captivated by the elegance of the finding and wondered if nitric oxide might do something in the brain. First let me describe the background.

Robert Furchgott, a pharmacologist at Downstate Medical Center in Brooklyn, had been studying blood vessel relaxation. The classic neurotransmitter acetylcholine relaxes blood vessels. To study this process directly, Furchgott “cleaned” his blood vessels, removing the inner endothelial layer to provide direct access of acetylcholine to the underlying muscle. When he did this, acetylcholine no longer elicited relaxation. Reasoning that something from the endothelial layer was critical, he restored it, and the relaxation returned. He concluded that the acetylcholine must have triggered the release from the endothelium of a chemical that causes blood vessel relaxation, which he dubbed “endothelial derived relaxing factor (EDRF)” (14). For a number of years numerous investigators strove to identify the substance, but it seemed to be extraordinarily labile and to have a variety of peculiar features. Identification of EDRF as NO might never have taken place except for the convergence of different lines of research. The pharmacologist Ferid Murad sought to know how nitroglycerin potently relaxes blood vessels to elicit its therapeutic actions in cardiac angina. He demonstrated that nitroglycerin must first be converted to NO as an active metabolic product (15). Louis Ignarro, a pharmacologist at UCLA, had been pursuing a similar line of investigation with similar conclusions. Simultaneously, his laboratory had been trying to identify EDRF. He had detected various similarities between EDRF and nitric oxide, such as antagonism of their actions by the dye methylene blue and their heme-dependent augmentation of the activity of the cyclic GMP-forming enzyme guanylyl cyclase. Emboldened by these clues, Ignarro devised experiments proving that EDRF and NO are identical in terms of their ability to influence contractions of a wide range of smooth muscle preparations as well as to influence the absorption spectrum of hemoglobin (16).

The Nobel Prize in Physiology and Medicine was awarded to Furchgott, Murad, and Ignarro in 1998. Their efforts reflect the Audacity Principle. When Furchgott carried out his work identifying EDRF, few biomedical scientists cared about the nuances of blood vessel relaxation. Furchgott’s paper describing EDRF lacked any evidence for its chemical structure, which rendered the enterprise somewhat dubious. The search for the identity of EDRF, which consumed many years, was fraught with major hurdles in seeking a molecule so labile as NO. But for the link to nitroglycerin, the search might well have failed. Most scientists would have dropped the project in favor of one with a greater guarantee of short-term success.

When I read about NO, its remarkable properties spawned the fantasy that this molecule might do something in the brain. The British biochemist John Garthwaite had published a paper showing that brain cultures could synthesize a molecule with the properties of EDRF (17). My M.D. Ph.D. student David Bredt and I decided that it was worth exploring the functions of

NO in the brain. We took lessons from what was already known about blood vessels. NO relaxes blood vessels by stimulating formation of the second messenger molecule cyclic GMP formation. In the brain the major excitatory neurotransmitter, glutamic acid, stimulates the enzyme guanylyl cyclase, which makes cyclic GMP. We wondered whether NO was involved. We knew that NO is formed from the amino acid arginine and that arginine derivatives act as inhibitors of the enzyme. One of these arginine derivatives prevented cyclic GMP formation in proportion to its ability to block the formation of NO. Clearly NO somehow mediated the actions of glutamic acid, generally regarded as the most prominent neurotransmitter in the brain. This convinced us that we were dealing with an area of importance for brain function.

What were we to do? We knew it was important to isolate and clone the enzyme that converts arginine to NO, but numerous other laboratories had already failed in this endeavor. First, it was notoriously difficult to monitor the conversion of arginine to NO, since the labile NO is not readily detected by most techniques. Existing assays to monitor the activity of the NO synthase enzyme were insensitive and cumbersome, which would have precluded the large number of assays required for enzyme purification. We decided to measure the conversion of radiolabeled arginine to the amino acid citrulline, which is formed as a by-product, and developed a simple ion exchange column procedure enabling us to conduct fifty assays in an hour.

Next was the challenge of purifying the enzyme. Why should we succeed where so many others had failed? In our first experiment we found what all other investigators had encountered. When one tried to purify a brain extract over an ion exchange column separating large numbers of fractions, all enzyme activity vanished. The enzyme seemed hopelessly unstable. David wondered whether the enzyme was in fact stable, but the purification procedure had separated out a crucial cofactor. When he reconstituted the test tube fractions, enzyme activity reappeared, establishing that something had been lost in the purification process. How would we purify and isolate the mysterious cofactor? One could presumably pursue the laborious process of determining the substance's properties and trying to isolate it, which could take months. David chose instead to guess. He knew that calcium played some role in augmenting NO formation. Calcium interacts with a large number of proteins, but the best characterized is a small protein called calmodulin, required for the activity of many enzymes. David added calmodulin, and enzyme activity returned (18).

The discovery of calmodulin as a crucial factor for NO synthase clarified certain mysteries. Acetylcholine must trigger the formation of nitric oxide very rapidly to mediate the normally rapid blood vessel reactivity. If NO were a neurotransmitter, a similarly swift activation of its formation would be required with each nerve impulse. We knew already that neuronal firing is as-

sociated with an immediate influx of calcium, and acetylcholine augments calcium levels inside blood vessel cells. For NO to be a neurotransmitter, its biosynthetic enzyme would have to be activated every time NO-containing nerves fired, because, unlike classical neurotransmitters, NO, a gas, couldn't exist in large storage pools in synaptic vesicles. The link to calmodulin resolved these dilemmas. NO can be formed "on-line" with nerve or neurotransmitter activity, as the associated rapid calcium entry activates its biosynthetic enzyme.

With NO synthase stabilized by calmodulin, David was able to purify the enzyme protein to homogeneity, obtain its amino acid sequence, and clone its gene (19). This led to the discrimination of three separate forms of the enzyme, a neuronal form, a blood vessel or endothelial form, and an inducible one that occurs in all cells of the body. Myriad advances in NO biology would not be possible without the cloned enzymes.

Establishing definitively that NO is a neurotransmitter did not employ the brain, so complex that it is the poorest preparation for clarifying neurotransmitter properties. The classic neurotransmitters acetylcholine and norepinephrine were all characterized in the peripheral nervous system many decades before their role in neurotransmission of the brain was established. In the case of NO, our ability to purify its biosynthetic enzyme enabled us to generate antibodies to NO synthase. With these antibodies we could visualize the enzyme by immunohistochemistry (20). First, we examined blood vessels and found the enzyme highly concentrated in the endothelial layer, providing definitive evidence that this is the place where NO is generated in response to acetylcholine. In peripheral organs we found the enzyme localized to neuronal structures. In the intestine, NO synthase occurs in the myenteric plexus of neurons, which regulates the contraction and relaxation that underlie digestive peristalsis. Since the 1940s scientists tried unsuccessfully to implicate the classic neurotransmitters acetylcholine and norepinephrine in peristaltic relaxation. They concluded that some other neurotransmitter mediates what they referred to as non-adrenergic, non-cholinergic (NANC) relaxation. Several laboratories simply added NO synthase inhibitors and observed a substantial reduction in NANC relaxation, indicating a transmitter role for nitric oxide. Our cloning of the gene for neuronal NO synthase led to the generation of mice with targeted deletion "knockout" of the gene. We showed that NANC relaxation was reduced about 50 percent in these gene knockout mice, consistent with the findings using enzyme inhibitors.

Even more dramatic than intestinal neuronal localizations of the enzyme was David's observation that the cavernous nerves that project to the penis are extremely enriched in NO synthase. The cavernous nerve is required for penile erection. This led us to collaborate with Dr. Arthur Burnett, a col-

league in the urology department. Burnett had developed an elegant system in which electrical stimulation of the cavernous nerves of intact rodents produces robust penile erection. NO synthase inhibitors abolished erection (21). Interestingly, penile erection involves a process analogous to blood vessel dilation. Erection takes place when the smooth muscle of the venous sinuses of the penis relaxes, enabling them to become engorged with blood. As with conventional blood vessels, engorgement of penile venous sinuses involves cyclic GMP. Cyclic GMP is rapidly degraded by the enzyme phosphodiesterase (PDE), and PDE inhibitors elevate cyclic GMP levels. When publications appeared establishing NO as a neurotransmitter of erection, scientists at the Pfizer drug company resurrected one of their PDE inhibitor drugs, which had been ineffective in treating angina. Their clinical trials validated the drug as a treatment for erectile dysfunction, and it was subsequently marketed as Viagra.

The dramatic properties of NO as a gaseous neurotransmitter led to a seemingly simple but not necessarily obvious question: "Might there be other gaseous neurotransmitters?" Neurotransmitters come in chemical classes. First were the biogenic amines such as acetylcholine, norepinephrine, dopamine, and serotonin. Next were amino acid neurotransmitters such as gamma-aminobutyric acid (GABA) and glutamic acid, followed by peptides such as the enkephalins, substance P, cholecystokinin, and many others. What other gases should we explore as possible neurotransmitters? Ethylene is a gas with important functions in plants, but we could find no evidence for a role in animal tissues. Ajay Verma, an M.D. Ph.D. student, suggested carbon monoxide (CO). He had noted in biochemistry textbooks that when the enzyme heme oxygenase degrades heme emerging from aging red blood cells, it breaks open the heme ring to form the green pigment biliverdin, which is rapidly reduced to the yellow pigment bilirubin. In the process, a one-carbon fragment is released as CO. Though this biochemical pathway had been well established for decades, neither I nor most of my colleagues were aware of it, presumably because nobody had ever given any thought to biological functions for CO.

We explored what was known of heme oxygenase (HO). The enzyme was first described in the spleen, where aging red blood cells congregate. Formation of the principal form of the enzyme is induced by heme and many other substances. We found a publication describing a second form of HO that emerged during purification of the classic form. This enzyme, designated HO2, was not inducible, and was hence of little interest to the hematologists who were the principal students of heme degradation. Ajay found HO2 highly concentrated in discrete areas of the brain in neurons with localizations closely resembling guanylyl cyclase, suggesting that CO, like NO, might normally stimulate cyclic GMP formation (22).

With antibodies to HO2, we visualized the enzyme by immunohistochemistry. In the intestine HO2 occurs in the same myenteric plexus neurons as neuronal NO synthase. Hence, NO and CO might well function as co-neurotransmitters. Definitive evidence that CO is a neurotransmitter came from our experiments with NANC relaxation of the intestine in HO2 knockout and/or neuronal NO synthase knockout mice. As mentioned already, the neuronal NO synthase knockout mice display a 50 percent decrease in NANC relaxation. The HO2 knockout mice also showed a 50 percent decline in the process (23). Subsequently we cross-bred the two gene knockout mice. Loss of both NO and CO generating systems led to virtual abolition of NANC relaxation. Thus, NO and CO are major neurotransmitters of the intestinal relaxation that mediates the peristaltic process so crucial for digesting food. Subsequent work has shown that NO and CO are likely neurotransmitters in multiple sites in the brain and throughout the body.

Many investigators would never have dreamed of suggesting that gases such as NO and CO might be neurotransmitters. Such a proposition would overturn myriad principles of neurotransmission as fundamental to the field as Newton's Laws are to physics. Here are some of the classical criteria for establishing a chemical as a neurotransmitter. Neurotransmitters must be stored in synaptic vesicles and released by a process called exocytosis, in which the walls of the synaptic vesicles fuse with the plasma membrane of the cell spewing out the transmitter molecule. Indeed, Bernard Katz's Nobel Prize was awarded for his experiments showing that neurotransmitters are released in little packets or quanta that emerge from synaptic vesicles. Neurotransmitters are classically assumed to act upon specific receptor sites, proteins on the external membrane of the adjacent neurons. For neurons to respond to rapid firing patterns, there must exist large storage pools of the neurotransmitter molecule to prevent a neuron from losing the ability to respond to neuronal stimulation.

Clearly NO and CO overturn these "rules." Neither can be stored in synaptic vesicles; they must be resynthesized with each new nerve impulse. As indicated above, calcium influx associated with neuronal depolarization activates neuronal NO synthase. Fairly recently, we found that a similar process takes place for HO2. NO and CO don't act upon classic receptor proteins on adjacent neurons. Instead, they diffuse into the adjacent cell and bind to guanylyl cyclase, activating it to form more cyclic GMP. More recently, it has been established that NO can also act by chemically modifying target proteins via a process called S-nitrosylation of cysteines in the proteins.

When we embarked on our studies of NO and then of CO as neurotransmitters, we were aware that they did not fit established paradigms. We knew that anything we did in this area would therefore be met with great

skepticism, if not derision. However, each experiment led to another with an inexorability that was irresistible. Such “disregard” for what others might think presumably reflects the Audacity Principle.

Conclusion

Perhaps the most audacious aspect of this essay is my chutzpah in titling it “The Audacity Principle.” Many distinguished students of the scientific discovery process have conducted scholarly studies, while my presentation is anecdotal and derived largely from personal experience. I do not claim to have identified the most crucial talent underlying important scientific advances. Rather, I felt it of interest to provoke thinking about the various factors that separate the scientific “men from boys.” Clearly the mentor-apprentice relationship is paramount. Scientists do not learn originality from a book any more than do artists and composers. Innate gifts are equally important, for only a small percentage of the students of great scientists manifest comparable greatness. The essence of those gifts is hard to divine, but originality, simplicity, and audaciousness overlying a bedrock of intellect seem to be consistent ingredients. Blending these qualities in just the right proportions appears to do the trick. I have emphasized audacity, because, in its absence, the other qualities fail. Needless to say, audacity on the part of unintelligent, uncreative individuals is disastrous. However, in the right mix, audacity can make all the difference.

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Index

Page numbers printed in boldface type refer to tables or figures.

- Acetaminophen (Tylenol), xxvi
- Acetophenazine (Tindal), 142–143
 - affinities for muscarinic cholinergic receptor binding, **142**
 - structure of, **140**
- Acetylcholine, 223
 - timeline research, **28**
- Acetylcholinesterase, 440
- Addiction, mechanisms of, 62–64
- Adenosine, dependence of lithium's reversal of carbachol block of, **190, 192**
- Adenovirus infection, 331–332
- Adenylyl cyclase, 98
 - bidirectional regulation, **221, 212**
- β -Adrenoceptors, 31
- Agarose binding, 375
- Aggression, 278, **279, 280, 281**
- Agonists. *See* Opiate agonists
- Akil, Huda, 30
- Alkylamino
 - affinities for muscarinic cholinergic receptor binding, **142**
 - structure of, **140**
- ALS. *See* Amyotrophic lateral sclerosis
- Alzheimer's disease, xxxiv
- Amino acids, xxxii
 - commentary, 291–295
 - determination of composition and sequence, 70–76, **75**
 - sequence of nitric oxide synthase, **264–265**
- D-Amino acids
 - analysis, 299
 - D-aspartate, neuronal and neuroendocrine roles, 297–312
 - commentary, 291–295
 - serine racemase, 313–327, **329–347**
- γ -Aminobutyric acid (GABA), xxviii, 210
 - [^3H]GABA, 31
- Amitriptyline
 - affinities for neurotransmitter receptor binding sites, **164, 165, 166**
 - comparison of affinities for WB-4101 binding sites, **149**
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, **172, 173**
 - depression with psychomotor agitation and, **151**
 - effect of long-term treatment on neurotransmitter receptor binding, **166, 167**
- AMPA receptor activation, 334, 336, **338–339**
- Amphetamines, 209
 - effect of chronic drug treatments on haloperidol binding and, **115**
- Amyotrophic lateral sclerosis (ALS), studies examining relationship of bilirubin to human disease, **389–390**
- Analgesics, xxvi

- Animal studies, 37, 100, 139–140, 143, 446. *See also* Inositol phosphates; Research
- for antischizophrenic drugs, 103–111
- behavioral abnormalities in mice
- lacking neuronal nitric oxide synthase, 277–287
 - aggressive behavior, 278, 279
 - defensive aggressive behavior, 278, 280
 - offensive aggressive behavior, 278, 281
 - sexual behavior, 282, 284–285
- capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
- distribution of autoradiographic grains labeling the opiate receptor, 60
- drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
- effect of long-term antidepressant treatment on neurotransmitter receptor binding, 166, 167
- effects of sodium on binding of opiate agonists and antagonists, 23
- effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- immunohistochemical mapping in rat central nervous system, 81–93
- morphine-like peptides in mammalian brain, 69–79
- opioid peptide enkephalin, 81–93
- regional localization of the morphine-like factor and opiate receptors, 62
- serine racemase, 313–327
- stereospecific [^3H]naloxone binding in various tissues, 14–15
- of tardive dyskinesia, 113–114
- Antagonists. *See* Opiate antagonists
- Antibodies, 374, 453
- production, 316
 - specificity test, 300, 300
- Anticholinergics, xxx
- Antidepressants. *See also* Tricyclic antidepressants
- affinities for neurotransmitter receptor binding sites, 164, 165, 166
 - long-term use, 163–170
 - effect on neurotransmitter receptor binding, 166, 167
 - spiperidol-labeled serotonin receptor binding and, 163–170
- Antioxidants, xxxiii
- studies examining relationship of bilirubin to human disease, 389–390
- Antiparkinsonian drugs, xxviii. *See also* individual drug names
- Antischizophrenic drugs, 103–111. *See also* individual drug names
- brain cholinergic receptors and, 137–146
 - phenothiazines and
 - butyrophenones for muscarinic cholinergic receptor binding, 142
 - phenothiazines for muscarinic cholinergic binding sites, 144
 - structures of clozapine and haloperidol, 141
 - structures of phenothiazines, 140
- chronic treatment with, 113–119
- comparison of affinities for haloperidol and dopamine binding sites, 106–107
- effect of chronic drug treatments on haloperidol binding, 115
- regional comparison of competition for haloperidol binding by, 108

- time course and dose dependence of increase in haloperidol binding after chronic haloperidol treatment, 116
- Apomorphine, 117
- Apoptosis, xxxiii, 185, 399–431, 427
- Archives of General Psychiatry*, xxvi
- Arginine, xviii, 250
 - pharmacology of excitatory amino acid-mediated enhancement, 252, 253
 - specificity of reversal of MeArg inhibition of cGMP accumulation, 255, 255
- Artane (trihexyphenidyl hydrochloride), 142
- D-Aspartate, xxxiii
 - antibody production and purification, 298
 - immunohistochemistry, 298–299
 - neuronal and neuroendocrine roles, 297–312
 - antibody specificity test, 300, 300
 - disposition in the pineal gland, 300, 302
 - inverse brain localizations, 302, 304
 - inverse localizations in P23 cerebellum, 304, 308
 - inverse localizations in the olfactory bulb, 304, 307
 - localization, 300, 301
 - localization in P23 septal neurons, 306
 - in the pituitary gland, 301, 303
- D-Aspartate oxidase (DAPOX)
 - cellular localization, 303, 305
 - inverse brain localizations, 302, 304
 - localization of D-aspartate, 299, 301
 - norepinephrine histochemistry and, 299
 - visualized in the pituitary and hypothalamic nuclei, 301, 303
- Atopic dermatitis, studies examining relationship of bilirubin to human disease, 389–390
- Atropine, drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
- Audacity principle, 439–455
- Autoradiography, 59
 - distribution of autoradiographic grains labeling the opiate receptor, 60
- Axelrod, Julius, xv, xxvi, xxvii, xxxiii–xxxiv, 439–455
- Azaperone, comparison of affinities for haloperidol and dopamine binding sites, 107
- Behavior
 - behavioral abnormalities in mice lacking neuronal nitric oxide synthase, 277–287
 - aggressive behavior, 278, 279
 - defensive aggressive behavior, 278, 280
 - offensive aggressive behavior, 278, 281
 - sexual behavior, 282, 284–285
 - role of nitric oxide, 246
 - sensitivity to apomorphine, 117
- “Belladonna” actions, 139
- Bennett, Jim, xxix
- Benperidol, comparison of affinities for haloperidol and dopamine binding sites, 106
- Benzodiazepines, 210
- Benzomorphans, 36–37
- Benztropine mesylate (Cogentin), xxviii, 142
- Berridge, Michael, xxxi, xxxii
- Bilirubin, xxxiii, 351, 353
 - benefits, 383–398
 - biliverdin reductase cycle, 385–387
 - clinical relevance, 387–392, 388–390
 - oxidation-reduction cycles, 384, 385

Bilirubin (*continued*)

- studies that examined the relationship to human disease, 389–390

Biliverdin reductase cycle, 385–387

Bloom, Floyd E., 30

Borko, Elaine, xxvi

Brain. *See also* Dopamine receptors;

- Receptor binding
- cholinergic receptors,
- antischizophrenic drugs and, 137–146

- chronic treatment with
- antischizophrenic drugs, 113–119

- colocalization of serine racemase, 324–325

- distribution of autoradiographic grains

- labeling the opiate receptor, 60

- distribution of nitric oxide synthase
- in, 244–245

- drug potencies in reducing
- stereospecific [^3H]naloxone binding, 12–13

- function of nitric oxide in, xviii–xix

- heterogeneous localization of
- second messenger systems, 219–221

- inhibition and potentiation by
- superoxide dismutase of
- NMDA-mediated increase in
- cGMP in cerebellar slices, 255, 257

- inverse localizations, 302, 304

- inverse localizations of D-aspartate, 304, 308

- localization of nitric oxide synthase, 269

- morphine-like peptides in
- mammals, 69–79

- nitric oxide synthase expression, 268

- normal function, 53–54

- opiate receptor in normal and drug
- altered brain function, 53–67

purification of calf brain

enkephalin, 71, 72

receptor binding in, 19–26

regional mapping, 58–59, 60

stereospecific [^3H]naloxone binding

in various tissues, 14–15

Bredt, David, xviii

Bromo-LSD, comparison of drug

potencies at serotonin receptors

with physiological actions, 174, 175

Bromperidol, comparison of affinities

for haloperidol and dopamine

binding sites, 106

Brown, Donald, xxiv–xxv

α -Bungarotoxin, 27–28

Bunney, William (Biff), xxix, 30

Burt, David, xviii

Bush, President George W., xxi

Butaclamol, comparison of affinities for

haloperidol and dopamine binding

sites, 106

Butyrophenones, 138, 103–111

affinities for muscarinic cholinergic

receptor binding, 142

structure of, 141

Calcium, 184

imaging, 333, 375–376

inhibition by inositol derivatives,

201, 201–204

purified inositol receptor in

reconstituted lipid vesicles,

231–239

release measurements, 372

tissue factors mediating

sensitivity of inositol, 204,

205

Calmodulin, 204, 450–451

cAMP. *See* Cyclic adenosine

monophosphate

Cancer

chemotherapy, xxxi

studies examining relationship of

bilirubin to human disease,

389–390

- Carbachol
concentration dependence of
reversal of block of adenosine,
190, 192
phosphoinositide-mediated
response, 190, 191
- Carbamazepine, 183
- Carbamylcholine, drug potencies in
reducing stereospecific
[³H]naloxone binding, 12–13
- Carbon monoxide (CO), 352–353
- Carlsson, Arvid, 447
- Caspase activity, 375
- CAT assay, 420
- Catechol-*O*-methyltransferase
(COMT), xxvi
discovery, 440
- Cell culture, 419
- Cell death, xxxiii, 185, 399–431, 427
- Cell structure, 314
- Cell synchronization, 430
- Central nervous system (CNS)
actions in brain diseases, 133–135
immunohistochemical mapping in
rat, 81–93
- cGMP. *See* Cyclic guanosine
monophosphate
- Chemotherapy, xxxi
- Chloride, opiate receptor function
and, 58
- Chlorimipramine
comparison of affinities for WB-
4101 binding sites, 149
depression with psychomotor
agitation and, 151
- Chlorpromazine (Thorazine), 142
affinities for muscarinic
cholinergic receptor
binding, 142, 144
affinities for neurotransmitter
receptor binding sites, 164,
165, 166
comparison of affinities for
haloperidol and dopamine
binding sites, 107
comparison of drug affinities with
drug inhibition of serotonin-
related behavior, 172, 173
effect of long-term treatment on
neurotransmitter receptor
binding, 166, 167
serum levels measured by
radioreceptor assay, 127
structure of, 140
therapeutic effects, 128
for treatment of schizophrenia, 139
- Choline, drug potencies in reducing
stereospecific [³H]naloxone
binding, 12–13
- Cinanserin
comparison of drug affinities
with drug inhibition of
serotonin-related behavior,
172, 173
comparison of drug potencies at
serotonin receptors with
physiological actions, 174, 175
- Citrulline
concentration–response
relationships for NMDA
stimulation and inhibition,
252, 254
stoichiometric inhibition, 252, 252
- Clofluprolol, comparison of affinities
for haloperidol and dopamine
binding sites, 106
- Cloning, 212–213, 314. *See also* Serine
racemase
for central nitric oxide actions, 244–
245
nitric oxide synthase and, 262, 264–
265
of Siah1 cDNA, 418
- Clozapine, xxx, 99–100
affinities for muscarinic
cholinergic receptor binding,
142, 144
comparison of affinities for
haloperidol and dopamine
binding sites, 107

Clozapine (*continued*)

- comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
- regional comparison of competition for haloperidol binding, 108
- structure of, 141

CNS. *See* Central nervous system

CO. *See* Carbon monoxide

Codeine, 446

- competition for [³H]naloxone binding, 32
- drug potencies in reducing stereospecific [³H]naloxone binding, 12–13

Cogentin (benztropine), xxviii, 142

Co-immunoprecipitation, 374, 419

Colchicine, drug potencies in reducing stereospecific [³H]naloxone binding, 12–13

Coleus forskohlii, 213

COMT. *See* Catechol-O-methyltransferase

Concanavalin A, 202

Coronary artery disease, studies examining relationship of bilirubin to human disease, 389–390

Coyle, Joseph, xxvii–xxviii

Creese, Ian, xviii, 30

Cuatrecasas, Pedro, xxix–xxx, 30

Cyclazocine

- differential receptor interactions, 55
- differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35
- effects of sodium on inhibition by opiate agonists and antagonists of stereo specific [³H] binding, 24

Cyclic adenosine monophosphate (cAMP), xxxi

Cyclic guanosine monophosphate (cGMP), xviii
addiction and, 63–64

Cyproheptadine

- comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
- comparison of drug potencies at serotonin receptors with physiological actions, 174, 175

Cytochrome c, xxxiii, 351

- agarose binding, 375
- binding in intact cells, 366, 367
- cytosolic calcium and, 366–369, 368, 370
- effects of cytochrome on homeostasis, 380
- interactions, 357, 358
- in response to STS treatment, 382
- translocation to endoplasmic-reticulum-binding, 359, 360–361, 362, 364, 365, 379

Cytochrome P450

- reductase, 261–275
- system, xxvi

Cytotoxicity, 420

Dansyl-Edman sequence
determination, 72

DAPOX. *See* D-Aspartate oxidase

Dawson, Ted, xviii–xix

DEAE-cellulose, 202

Delta opioid peptide (DOP), 37
timeline research, 28

Deoxycholate, 21

Deprenyl, xxxv

Depression, 147. *See also* Tricyclic antidepressants
with psychomotor agitation, 151

Desipramine

- affinities for neurotransmitter receptor binding sites, 164, 165, 166
- comparison of affinities for WB-4101 binding sites, 149
- comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173

- depression with psychomotor agitation and, 151
- effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- Detergents, 197–199, 199, 200, 201
- Dev, Parvati, 30
- Dextrophan, 15–16
 - competition for [^3H]naloxone binding, 32
 - drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
- Diacylglycerol (DAG), 214, 215, 216, 218
- Diazepam, 160
- Dibenzodiazepine
 - affinities for muscarinic cholinergic receptor binding, 142
 - structure of, 141
- [^3H]Dihydroalprenolol, 31
- Dihydromorphine
 - differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - effects of sodium on binding of opiate agonists and antagonists, 23
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- Diprenorphine
 - differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
 - regional mapping and, 59
- Divalent cations, opiate receptor function and, 57
- Dole, Vincent P., 30
- DOP. *See* Delta opioid peptide
- [^3H]Dopamine, xviii, 31
- Dopamine receptors
 - antischizophrenic drugs and, 103–111
 - chronic treatment of antischizophrenic drugs, 113–119
 - commentary, 97–101
 - comparison of affinities for binding sites, 107
 - dopamine-based radioreceptor assay for neuroleptics, 125–128, 127
 - “hypothesis,” 137–138
 - identification, xviii, 124
 - in postmortem brains of schizophrenic patients, 124–125, 125
 - regional comparison of competition for haloperidol binding, 108
 - schizophrenia and, 121–130
 - types of, 122–124, 123
- Doxepin, comparison of affinities for WB-4101 binding sites, 149
- Droperidol, comparison of affinities for haloperidol and dopamine binding sites, 106
- Drug–receptor interactions, 134
- Drugs. *See also* Tricyclic
 - antidepressants; individual drug names
 - antiparkinsonian, xxviii
 - antischizophrenic. *See* Antischizophrenic drugs
 - capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 - clinical significance of serotonin receptor subtype identification, 157–161
 - commentaries, 133–135, 157–161
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173

Drugs (*continued*)

- comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
- competition for [³H]naloxone binding, 32
- development, 33–35
- differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35
- effect of chronic drug treatments on haloperidol binding and, 115
- effects of sodium on binding of opiate agonists and antagonists, 23
- effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [³H] binding, 24
- opiate receptor in normal and drug altered brain function, 53–67
- pharmacology of excitatory amino acid-mediated enhancement of arginine to citrulline conversion, 252, 253
- potencies in reducing stereospecific [³H]naloxone binding, 12–13
- serum levels with neuroleptics, 127
- time course and dose dependence of increase in haloperidol binding after chronic haloperidol treatment, 116

EDRF *See* Endothelium-derived relaxing factor

EDTA (ethylene diamine tetra-acetic acid), 57

EGTA (ethylene glycol tetra-acetic acid), 57

Endorphins, xxx, xxxii

Endothelium-derived relaxing factor (EDRF), 261, 449

[³H]Enkephalin, 37

Enkephalins, xxx, xxxii, xxxiv, 38–39
commentary, 49–52

- identification of, 37
- morphine-like peptides in mammalian brain, 69–79
- assessment of enkephalin purity and determination of amino acid composition and sequence, 71–76, 75
- determination of amino acid composition and sequence, 70–71
- extraction and purification of enkephalin, 70
- interactions of natural and synthetic enkephalins with the opiate receptor, 76, 77
- purification and isolation of enkephalin, 71, 72, 73, 74
- opiate receptor in normal and drug altered brain function, 53–67
- opiate peptide enkephalin, 81–93
- purification, 71, 73, 74
- second messenger systems and, 213, 214

Enzymes, 197, 198

- enzymatic modulation of nitric oxide, 245–246

[³H]Ethylketocyclazocine, 37

Etorphine, 31

- differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35

- effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [³H] binding, 24

Excitotoxicity, 185

- nitric oxide and, 245

Fishman, Mark, xix

FK-506, xx

Fluanisone, comparison of affinities for haloperidol and dopamine binding sites, 106

Fluoxetine (Prozac), xxix

- affinities for neurotransmitter receptor binding sites, 164, 165, 166
- effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- α -Flupenthixol, comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
- Fluphenazine (Prolixin; Permitil), 142–143
 - affinities for muscarinic cholinergic receptor binding, 142
 - comparison of affinities for haloperidol and dopamine binding sites, 106
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
 - effect of chronic drug treatments on haloperidol binding and, 115
 - regional comparison of competition for haloperidol binding, 108
 - serum levels measured by radioreceptor assay, 127
 - structure of, 140
- Fluspirilene, comparison of affinities for haloperidol and dopamine binding sites, 106
- FRET, 375
- Freud, Sigmund, xxiv
- Fuller, Ray, xxix
- Furchgott, Robert, 449
- GABA. *See* γ -Aminobutyric acid
- [^3H]GABA, 31
- GAPDH. *See* Glyceraldehyde-3-phosphate dehydrogenase
- Genes, 271
- Glutamates, xviii, xxxiii
 - effect of nitric oxide on function of, 244
- Glutathione (GSH), oxidation-reduction cycles, 384, 385
- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), xxxiii, 351
 - Siah1 mediation, 400–402, 401, 402
 - stabilization of Siah1, 402–405, 404
 - subcellular distribution, 425–426
- Glycine, xvii
 - opiate receptor function and, 58
- Goldstein, Avram, xxix, 30
- G-proteins, 4, 5
 - development, 33
 - potential actions of lithium through inositol phosphatases and G-proteins, 221–224
 - second messenger systems and, 211–213, 212, 214
 - timeline research, 28
- Granule cell migration assay, 332
 - on SR-transfected HEK-293 cells, 333
- Green, Alan, xxvii–xxviii
- GRIP PDZ-6, 331
- GSH. *See* Glutathione
- GST pulldown assay, 372, 418–419
- GTP. *See* Guanosine triphosphate
- Guanosine triphosphate (GTP), 122–123
- Haldol. *See* Haloperidol
- Hall, Robert D., 30
- Haloperidol (Haldol), xxx, 97–98, 104
 - affinities for muscarinic cholinergic receptor binding, 142
 - affinities for neurotransmitter receptor binding sites, 164, 165, 166
 - comparison of affinities for binding sites, 107
 - comparison of affinities for haloperidol and dopamine binding sites, 106
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173

Haloperidol (*continued*)

- effect of chronic drug treatments and, 114, 115
- effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- regional comparison of competition for haloperidol binding by antischizophrenic drugs, 108
- serum levels measured by radioreceptor assay, 127
- structure of, 141
- time course and dose dependence of increase in haloperidol binding after chronic haloperidol treatment, 116
- for treatment of schizophrenia, 139

[³H]Haloperidol, xviii, 31

Hemoglobin, inhibition and potentiation by superoxide dismutase of NMDA-mediated increase in cGMP in cerebellar slices, 255, 257

Heparin, 39

- inhibition by inositol derivatives, 201, 201–204

Heparin-agarose, 202

Heroin, xxix, 55, 446

Herz, Albert, 30

Histamine

- drug potencies in reducing stereospecific [³H]naloxone binding, 12–13
- second messenger systems and, 213, 214

Homsy, Yvonne M., 30

5-HT. *See* Serotonin

Hughes, John, 30, xxx

Huntington's disease, xix, xxxiv

3-Hydroxy-*N*-allylmorphinan (levallorphan)

- capacity to increase stereospecific [³H]dihydromorphine binding, 20–21

competition for [³H]naloxone binding, 32

differential receptor interactions, 55

differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35

drug potencies in reducing stereospecific [³H]naloxone binding, 12–13

effects of sodium on binding of opiate agonists and antagonists, 23

effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [³H] binding, 24

Imipramine

affinities for neurotransmitter receptor binding sites, 164, 165, 166

comparison of affinities for WB-4101 binding sites, 149

depression with psychomotor agitation and, 151

effect of long-term treatment on neurotransmitter receptor binding, 166, 167

Immunophilins, xix–xx

Inositol phosphates, actions of lithium and. *See also* Inositol 1,4,5-trisphosphate receptor; Phosphoinositide system

commentary, 181–187

lithium and phosphoinositide-mediated cholinergic response, 189–194

metabolism, 214, 215

potential actions of lithium through inositol phosphatases and G-proteins, 221–224

purified IP₃ receptor and calcium flux in reconstituted lipid vesicles, 231–239

calcium flux and, 233, 236

- concentration–response
 - relationships, 233, 235
- reconstitution, 233, 234
- time course, 233, 237
- second messenger systems and
 - psychoactive drug action, 209–229
- solubilization, purification, and
 - characterization of IP₃ receptor, 195–207
 - experimental procedures, 196–197
 - results, 197–204
 - effects of reagents and enzymes, 197, 198
 - properties of the purified receptor protein, 201, 201–204
 - purification of binding site, 199–201, 203
 - solubilization of binding sites by detergents, 199, 200, 201, 197–199
 - tissue factors mediating the sensitivity of binding to calcium, 204, 205
- Inositol 1,4,5-trisphosphate (IP₃)
 - receptor, xvi–xvii, xix–xx, xxxi
 - regulation, 216, 217
- InsP₃. *See* Inositol 1,4,5-trisphosphate (IP₃) receptor
- Insulin receptors, timeline research, 28
- IP₃. *See* Inositol 1,4,5-trisphosphate (IP₃) receptor
- Iprindole
 - affinities for neurotransmitter receptor binding sites, 164, 165, 166
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
 - effect of long-term treatment on neurotransmitter receptor binding, 166, 167
 - IQ, 436
 - Iversen, Leslie, xxviii, 30
- Jacobson, Arthur E., 30
- Jaffe, Jerome, xxix
- Johns Hopkins School of Medicine, xxvii, xxix
- Johnson, L. Everett, 30
- Journal of Abnormal Psychology*, xxvi
- Journal of Biological Chemistry*, xxv
- Kaiser Foundation Hospital, xxvi
- Kanigel, Robert, 444
- Kappa opioid peptide (KOP), 37
 - clinical trials, 40
 - timeline research, 28
- Ketocyclazocine, 37
- Kety, Seymour, xxiv–xxv
- KOP. *See* Kappa opioid peptide
- Kosterlitz, Hans, xxx, 30
- Kramer, Peter, xxix
- Kuhar, Michael, xxviii
- Lasker Award, xxi
- Leu-enkephalin, 69–79
- Levallorphan (3-Hydroxy-N-allylmorphinan), 20–21
 - competition for [³H]naloxone binding, 32
 - differential receptor interactions, 55
 - differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35
 - drug potencies in reducing stereospecific [³H]naloxone binding, 12–13
 - effects of sodium on binding of opiate agonists and antagonists, 23
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [³H] binding, 24

- Levorphanol, 15–16
 capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 competition for [^3H]naloxone binding, 32
 differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 effects of sodium on binding of opiate agonists and antagonists, 23
 effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- Listening to Prozac* (Kramer), xxix
- Lisuride, comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
- Lithium, xxxi
 cholinergic response, 189–194
 to carbachol, 190, 191
 clinical therapeutic effects, 182
 concentration dependence of reversal of the carbachol block of adenosine, 190, 192
 potential actions of lithium through inositol phosphatases and G-proteins, 221–224
- Logan, Bill, xxix
- Loh, Horace, 30
- Lysergic acid diethylamide (LSD)
 D-LSD, comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
 [^3H]lysergic acid diethylamide (LSD), 31
- Mandell, Arnold J., xxix, 30
- Mansky, Peter A., 30
- MAO inhibitors. *See* Monoamine oxidase inhibitors
- Mapping
 immunohistochemical mapping in rat central nervous system, 81–93
 of opiate receptors, 58–59, 60
- Martin, William R., 30
- Matthyse, Steven, 30
- Mayer, David J., 30
- MeArg. *See* N^0 -monomethyl-L-arginine
- Medical Economics*, xxiii
- Meldolesi, Jacopo, xx
- Mellaril. *See* Thioridazine
- Meperidine
 differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- Mesoridazine, serum levels measured by radioreceptor assay, 126, 127
- Met-enkephalin, 69–79
- Metergoline
 comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
 comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
- Methadone
 competition for [^3H]naloxone binding, 32
 drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
- Methiothepin, comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
- Methysergide
 comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173

- comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
- effect of long-term drug treatment on neurotransmitter receptor binding, 167, 167
- Mianserin, comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
- Michaelis-Menten kinetics, xxviii
- Models
 - of addiction, 63–64
 - for D-serine and glutamate regulation of cerebellar granule cell migration, 339–340, 344
 - of opiate receptor function, 56–58
 - for side effects of antischizophrenic drugs, 143–144
- Molindone, serum levels measured by radioreceptor assay, 127
- Monoamine oxidase (MAO) inhibitors, 209
 - effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- MOP. *See* Mu opioid peptide
- Moperone, comparison of affinities for haloperidol and dopamine binding sites, 106
- Morphine, xxx, 36–37, 448
 - capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 - competition for [^3H]naloxone binding, 32
 - drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
 - opiate receptor function and, 57
- Morphine-like factor, 61–62, 62. *See also* Enkephalins
- Mu opioid peptide (MOP), 37
 - timeline research, 28
- Musacchio, Jose M., 30
- NADPH diaphorase, xix
- Nalorphine, 36–37
 - capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 - competition for [^3H]naloxone binding, 32
 - differential receptor interactions, 55
 - differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
 - effects of sodium on binding of opiate agonists and antagonists, 23
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
 - research timeline, 24
- Naloxone, 447
 - addiction and, 63–64
 - capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 - competition for [^3H]naloxone binding, 32
 - differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
 - effects of sodium on binding of opiate agonists and antagonists, 23
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24

- Naloxone (*continued*)
 inhibition of binding by enkephalin, 77
 $[^3\text{H}]$ naloxone, 4–5, 37
- Naltrexone
 differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 effects of sodium on inhibition by opiate agonists and antagonists of stereospecific $[^3\text{H}]$ binding, 24
- NANC. *See* Non-adrenergic, non-cholinergic relaxation
- National Academy of Sciences, xxi
- National Institute on Drug Abuse, xxix
- National Institutes of Health (NIH), xxv, xxiv
- National Medal of Science, xxi
- Nature*, 4, 50, 51
- Nervous tissue, opiate receptors and, 9–17
- Neural messengers
 bilirubin benefits, 383–398
 commentary, 351–354
 cytochrome *c*, IP_3 receptors, and calcium-dependent apoptosis, 355–382
 during apoptosis, 357–365, 360–361, 362, 364, 365, 367, function altering, 357, 358
 methods, 372–376
 S-nitrosylated GAPDH, apoptotic cell death, and Siah1 binding, 399–431
- Neuroleptics, xxx, xxxi. *See also*
 individual drug names
 affinities for neurotransmitter receptor binding sites, 164, 165, 166
 chronic treatment with antischizophrenic drugs, 113–119
 commentary, 97–101
 dopamine-based radioreceptor assay for, 125–128, 127
 effect of long-term treatment on neurotransmitter receptor binding, 166, 167
 schizophrenia and, 103–111, 121–130
 with sedative properties, 152
 serum levels, 127
- Neuroscience Research Program (NRP), 29, 30
- Neurotransmitter receptors
 effect of long-term antidepressant use and, 166, 167
 labeling with radioligands, xvii–xviii
- Nicotinic acetylcholine, timeline research, 28
- NIH. *See* National Institutes of Health
- Nitric oxide (NO), xvii
 cloning and properties of neuronal nitric oxide synthase, 245–246
 enzymatic modulation, 245–246
 homology to other proteins, 246
 protein properties, 245
 commentary, 243–248
- Nitric oxide synthase (NOS), xviii–xix
 behavioral abnormalities in mice
 lacking neuronal nitric oxide synthase, 277–287
 aggressive behavior, 278, 279
 defensive aggressive behavior, 278, 280
 offensive aggressive behavior, 278, 281
 sexual behavior, 282, 284–285
 catalytic activity, 267, 263
 cloning, 264–265, 262
 cytochrome P-450 reductase, 261–275
 cloning, 262, 264–265
 early observations, 243–244
 evidence for central nitric oxide actions, 244–245
 distribution of nitric oxide synthase in brain, 244–245
 effect on glutamate function, 244

- nitric oxide and excitotoxicity, 245
- functional expression, 262–263, 266, 267
- function in the brain, xviii–xix
- gene family, 271
- glutamate and cerebellar cGMP levels, 249–260
- materials and methods, 250–251
 - cGMP levels and citrulline formation in slices, 251
 - determination of nitric oxide, 250
 - materials, 250
 - preparation of brain slices, 250–251
 - results, 251–256, 252, 253, 254, 255, 256
 - stoichiometric inhibition, 252, 252
- history, xxxii–xxxiii
- mRNA expression, 263–265, 268, 269
- primary structure, 265–271, 270–271
- role in behavior, 246
- sequence alignment with cofactor binding regions, 270–271
- Nixon, President Richard, xxix, 29–30
- NMDA. *See* N-methyl-D-aspartate receptor
- N-methyl-D-aspartate (NMDA)
 - receptor, xvii, xx–xxi, xxxiii, 292–294
 - concentration–response relationships for stimulation and inhibition of citrulline, 252, 254
 - reversal of MeArg inhibition of NMDA-mediated enhancement of cGMP, 255, 255
- NO. *See* Nitric oxide
- Nobel Prize, 441, 444, 449
- Nociceptin/orphanin FQ (NOP)
 - receptor, timeline research, 28
- Non-adrenergic, non-cholinergic (NANC) relaxation, 451–453
- NOP. *See* Nociceptin/orphanin FQ receptor
- Noradrenergic receptor binding sites, tricyclic antidepressants and, 147–153
- Norepinephrine, xxviii
 - DAPOX histochemistry and, 299
 - drug potencies in reducing stereospecific [³H]naloxone binding, 12–13
- Nortriptyline
 - affinities for neurotransmitter receptor binding sites, 164, 165, 166
 - comparison of affinities for WB-4101 binding sites, 149
 - depression with psychomotor agitation and, 151
- NOS. *See* Nitric oxide synthase
- NRP. *See* Neuroscience Research Program
- N^ω-monomethyl-L-arginine (MeArg), 250
- Olfactory bulb, inverse localizations of D-aspartate, 304, 307
- Ondansetron (Zofran), xxxi
- Opiate agonists, 19–26
 - capacity to increase stereospecific [³H]dihydromorphine binding, 20–21
 - differential receptor interactions of antagonists and, 55–56
 - differentiating agonists and antagonists, 32–35, 32, 34
 - differentiation of opioid agonist and antagonist receptor interactions by Na⁺ ions, 34–35
 - effects of sodium on binding of opiate agonists and antagonists, 23
 - historical review, 27–46

- Opiate agonists (*continued*)
 non-addicting, 39–40
 versus receptor agonist, 4
- Opiate antagonists, 19–26
 capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 differential receptor interactions of opiate agonists and, 55–56
 differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 differentiation of agonists and antagonists, 32–35, 32, 34
 effects of sodium on binding of opiate agonists and antagonists, 23
 historical review, 27–46
 hypersensitivity to, 63–64
- Opiate receptors
 commentary, 3–7
 differentiation of agonists and antagonists, 32, 32–35, 34
 discovery, 6
 distribution, 86, 87, 89
 historical review, 27–46
 interactions of natural and synthetic enkephalins with, 76, 77
 multiple receptors development, 36–38
 in nervous tissue, 9–17
 drug potencies, 12–13
 in normal and drug altered brain function, 53–67
 demonstration, 54–55
 differential receptor interactions of opiate agonists and antagonists, 55–56
 endogenous morphine-like factor, 61–62, 62
 mechanisms of addiction, 62–64
 model of opiate receptor function, 56–58
 regional mapping, 58–59, 60
 radioligand binding methodologies, 3–7
 receptor binding in the brain, 19–26
 receptor localization, 35–36
 regional localization of the morphine-like factor and opiate receptors, 62
 timeline of research, 28
- Opioid peptide enkephalin, immunohistochemical mapping, 81–93
 antisera preparation, 82
 immunofluorescence, 82–83
 results, 83–90, 84, 85, 86, 87, 89
- Opioid peptide neurotransmitters, 38–39. *See also* Endorphins
 functions of, 40
- Oxotremorine-M, 189–190
- Oxymorphone
 capacity to increase stereospecific [^3H]dihydromorphine binding, 20–21
 differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 effects of sodium on binding of opiate agonists and antagonists, 23
 effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- P23, D-aspartate localization, 306
- Papas, Sophocles, xxiv
- Pargyline, effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- Parkinson's disease, xxxiv, 139, 447
- Pasternak, Gavril W., 30
- PDA. *See* Phorbol 12,13-diacetate
- PDE. *See* Phosphodiesterase
- Penfluridol, comparison of affinities for haloperidol and dopamine binding sites, 107

- Pentapeptides, 38
- Pentazocine
- competition for [^3H]naloxone binding, 32
 - differential receptor interactions, 55–56
 - differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - drug potencies in reducing stereospecific [^3H]naloxone binding, 12–13
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- Peptides, 37
- morphine-like peptides in mammalian brain, 69–79
- Peripheral vascular disease, studies
- examining relationship of bilirubin to human disease, 389–390
- Permitil. *See* Fluphenazine
- Perphenazine (Trilafon), 143
- affinities for muscarinic cholinergic receptor binding, 142
 - structure of, 140
 - for treatment of schizophrenia, 139
- Pert, Agu, 30
- Pert, Candace D., xvii–xviii, 30
- PET. *See* Positron emission tomography
- Phenazocine
- differentiation of opioid agonist and antagonist receptor interactions by Na^+ ions, 34–35
 - effects of sodium on inhibition by opiate agonists and antagonists of stereospecific [^3H] binding, 24
- Phenobarbital, drug potencies in
- reducing stereospecific [^3H]naloxone binding, 12–13
- Phenothiazines, 103–111, 138
- affinities for muscarinic cholinergic receptor binding, 142, 144
 - structures of, 140
- Phorbol 12,13-diacetate (PDA), 190–191
- Phosphodiesterase (PDE), 452
- Phosphoinositide (PI) system, 184, 213–218, 214, 215, 216, 217
- cholinergic response, 189–194
 - receptor-mediated, 214, 216
- PI. *See* Phosphoinositide system
- Pimozide
- comparison of affinities for haloperidol and dopamine binding sites, 106
 - regional comparison of competition for haloperidol binding, 108
- Pineal gland, D-aspartate disposition in, 300, 302
- Pipamperone, 105
- comparison of affinities for haloperidol and dopamine binding sites, 107
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173
- Piperazine
- affinities for muscarinic cholinergic receptor binding, 142
 - structure of, 140
- Piperidine
- affinities for muscarinic cholinergic receptor binding, 142
 - structure of, 140
- Pituitary gland, D-aspartate in, 303, 301
- PNAS, 51
- POMC. *See* Pro-opiomelanocortin
- Positron emission tomography (PET), 36
- Prolixin. *See* Fluphenazine
- Promazine (Sparine), 105, 142
- affinities for muscarinic cholinergic receptor binding, 142

- Promazine (*continued*)
 comparison of affinities for
 haloperidol and dopamine
 binding sites, 107
 structure of, 140
- Promethazine, 105
 comparison of affinities for
 haloperidol and dopamine
 binding sites, 107
 comparison of drug affinities with
 drug inhibition of serotonin-
 related behavior, 172, 173
 effect of chronic drug treatments on
 haloperidol binding and, 115
- Pro-opiomelanocortin (POMC), 39
- Propoxyphene
 competition for [^3H]naloxone
 binding, 32
 differentiation of opioid agonist and
 antagonist receptor interactions
 by Na^+ ions, 34–35
 drug potencies in reducing
 stereospecific [^3H]naloxone
 binding, 12–13
 effects of sodium on inhibition by
 opiate agonists and antagonists
 of stereospecific [^3H] binding,
 24
- Protein kinase C, 184, 218
- Proteins
 depletion, 429
 determination of, 197
 nitric oxide and, 245
 opiate receptor function and, 57
 second messenger systems and, 218
- Protriptyline
 comparison of affinities for WB-
 4101 binding sites, 149
 depression with psychomotor
 agitation and, 151
- Prozac (fluoxetine), xxix
- Psychoactive drugs, xvii–xviii
- QNB. *See* 3-Quinuclidinyl benzilate
- Quay, Wilbur, xxvii
- 3-Quinuclidinyl benzilate (QNB), 141
 [^3H]Quinuclidinyl benzilate, xvii–xviii,
 31
- Radiolabeled receptor ligand binding,
 97–101. *See also* Dopamine
 receptors; Neuroleptics
- Radioligands
 binding methodologies, 3–7
 labeling, xvii–xviii
- Radioreceptor assays, 126, 127
- Receptor agonist
 versus antagonist, 4, 35–36
 development, 36
 timeline research, 28
- Receptor binding. *See also* Brain
 in the brain, 19–26
 tricyclic antidepressants and, 147–
 153
- Research. *See also* Animal studies
 funding, 29–30
 participants in the Neuroscience
 Research Program Workshop
 (1974), 29, 30
 timeline of opioid receptor research,
 28
- Reserpine, 209
 effect of chronic drug treatments
 on haloperidol binding and,
 115
- Retinopathy of prematurity, studies
 examining relationship of bilirubin
 to human disease, 389–390
- Reversible ligand binding, xvi
- Roberts, Eugene, xxvii
- Rogers, Carl, 443
- Rosenthal, David, xxv
- Ross, Christopher, xx
- Schizophrenia, 100–101
 acute, xxv
 amino acid metabolism, xxv
 antischizophrenic drugs, 103–111
 chronic, xxv
 dopamine receptors and, 121–130

- dopamine receptors in postmortem brains of patients with, 124–125, 125
- neuroleptics and, 121–130
- Schmitt, Francis O., 30
- Schneider, Diana, 30
- Schwenk, Harriet, 30
- Science
 - commentary, audacity principle, 435–438
 - creative science and scientists, 435–438
 - disruptive, 291–295
 - gases as neurotransmitters, 448–454
 - lineage, 443–444
 - mentorship, 443–444
 - receptors, 444–448
- Science*, 50, 157
- SDS-PAGE analysis, 200–201, 203, 205, 232, 234
- Secondary amines, 150
 - affinities for neurotransmitter receptor binding sites, 164, 165, 166
 - comparison of affinities for WB-4101 binding sites, 149
- Second messenger systems, 209–229
 - cAMP and GTP binding proteins, 211–213, 212
 - heterogeneous localization in the brain, 219–221
 - phosphoinositide system, 213–218, 214, 215, 216, 217
 - potential actions of lithium through inositol phosphatases and G-proteins, 221–224
- Segovia, Andrés, xxiv
- Sephacryl S-400, 202
- D-Serine, xvii, xx–xxi, xxxii–xxxiii
 - synthesis, 331
- Serine racemase
 - adenovirus infection, 331–332
 - AMPA receptor activation, 334, 336, 338–339
 - antibody production, 316
 - calcium imaging, 333
 - cell culture and transfection, 314
 - cloning, 314
 - explant culture preparation, 332–333
 - granule cell migration assay, 332, 337–338, 342–343
 - granule cell migration on SR-transfected HEK-293 cells, 333
- GRIP PDZ-6 recombinant adenovirus generation, 331
- immunocytochemistry, 315, 331
- immunohistochemistry, 316
- model for D-serine and glutamate regulation of cerebellar granule cell migration, 339–340, 344
- mouse adenoviral infection and BrdUrd labeling, 332
- NMDA neurotransmission, 313–327
- primary cultures, 315–316
- protein binding assays, 330–331
- results, 316–321, 322, 320–321, 322, 324–325, 334–339
- D-serine synthesis, 314–315, 331
- SR and GRIP binding interactions and colocalization, 335, 334
- viral infection with GRIP, 340, 336–337
- yeast two-hybrid screening, 330
- Serotonin (5-HT), xxvii, 39
 - comparison of drug potencies at serotonin receptors with physiological actions, 174, 175
 - drug potencies in reducing stereospecific [³H]naloxone binding, 12–13
 - 5-HT₁, xxxi
 - 5-HT₃, xxxi
- Serotonin antagonists, effect of long-term treatment on neurotransmitter receptor binding, 166, 167
- Serotonin receptor binding
 - comparison of drug affinities with drug inhibition of serotonin-related behavior, 172, 173

- Serotonin receptor binding (*continued*)
 comparison of drug potencies at
 serotonin receptors with
 physiological actions, 174, 175
 with different physiological
 functions, 171–177
 long-term antidepressant use and,
 163–170
- Serotonin receptor subtype
 identification, 157–161
- Serotonin selective reuptake inhibitors
 (SSRIs), xxix. *See also* specific
 agents
- Sexual behavior, 282, 284–285
- Siah1 binding, 399–431
 cytotoxic actions, 413, 416–417
 nuclear translocation of GAPDH,
 400–413
 pulse-chase assay, 419–420
 S-nitrosylation, 405–407, 406, 408
- Signal transduction, xix–xx
- Simon, Eric J., 30
- snake toxin, 27–28
- S-nitrosylated GAPDH
 apoptotic cell death, and Siah1
 binding, 399–431
 nuclear translocation and cell death,
 409–412, 410–411, 427, 428
- S-nitrosylation biotin switch assay, 420
- Snyder, Solomon H., 30
- SOD. *See* Superoxide dismutase
- Sodium
 differential receptor interactions, 56
 effects on binding of opiate agonists
 and antagonists, 23
- Sodium dodecyl sulfate, 21
- Sparine. *See* Promazine
- [³H]Sipiperone, 31
- Spiroperidol, 105, 125
 comparison of affinities for
 haloperidol and dopamine
 binding sites, 106
 comparison of drug affinities with
 drug inhibition of serotonin-
 related behavior, 172, 173
 dopamine receptors in the brain
 and, 122
 long-term antidepressant use and,
 163–170
- SSRIs. *See* Serotonin selective reuptake
 inhibitors
- Staurosporine, 381
- Steiner, Joseph, xx
- Stelazine. *See* Trifluoperazine
- Stroke, xix
- [³H]Strychnine, xvii, 31
- Superoxide dismutase (SOD),
 inhibition and potentiation by
 superoxide dismutase of NMDA-
 mediated increase in cGMP in
 cerebellar slices, 255, 257
- Sweet, William H., 30
- Synaptosomes, 31
- Takemori, A. E., 30
- Tardive dyskinesia, 113–114
- Terenius, Lars, 30
- Tertiary amine tricyclics, 148, 149
 affinities for neurotransmitter
 receptor binding sites, 164,
 165, 166
 comparison of affinities for WB-
 4101 binding sites, 149
- Thioridazine (Mellaril), 99, 142
 affinities for muscarinic cholinergic
 receptor binding, 142, 144
 comparison of affinities for
 haloperidol and dopamine
 binding sites, 107
 comparison of drug affinities with
 drug inhibition of serotonin-
 related behavior, 172, 173
 plasma levels, 126
 regional comparison of competition
 for haloperidol binding,
 108
 serum levels measured by
 radioreceptor assay, 127
 structure of, 140
 therapeutic effects, 128

- Thiothixene, serum levels measured by radioreceptor assay, 127
- cis*-Thiothixene, comparison of affinities for haloperidol and dopamine binding sites, 106
- Thorazine. *See* Chlorpromazine
- Tindal. *See* Acetophenazine
- Transfectin, 314
- Tricyclic antidepressants, xviii. *See also* Antidepressants
- comparison of affinities for WB-4101 binding sites, 149
 - depression with psychomotor agitation, 151
 - effect of long-term treatment on neurotransmitter receptor binding, 166, 167
 - psychomotor activation, 150
 - therapeutic effects, 150–151
 - therapeutic properties and affinity for α -noradrenergic receptor binding sites in the brain, 147–153
- Trifluoperazine (Stelazine), 143
- affinities for muscarinic cholinergic receptor binding, 142, 144
 - comparison of affinities for haloperidol and dopamine binding sites, 106
 - serum levels measured by radioreceptor assay, 127
 - structure of, 140
 - for treatment of schizophrenia, 139
- Trifluoperidol, comparison of affinities for haloperidol and dopamine binding sites, 106
- Triflupromazine (Vesprin), 142
- affinities for muscarinic cholinergic receptor binding, 142
 - comparison of affinities for haloperidol and dopamine binding sites, 106
 - structure of, 140
- Trihexyphenidyl hydrochloride (Artane), 142
- Trilafon. *See* Perphenazine
- Triton-X 100, 19, 21, 202, 232
- TUNEL assay, 430
- Tylenol (acetaminophen), xxvi
- Tyr-Gly Gly Phe-Leu, 69–79, 75
- Tyr-Gly Gly Phe-Met, 69–79, 75
- Ubiquitination assay, 430
- U50488H, 38
- Valproate, 183
- Vesprin. *See* Triflupromazine
- Viral infection with GRIP, 336–337, 340
- Von Euler, Ulf, 441
- Warburg, Otto, 444
- Way, E. Leong, 30
- Whittaker, Victor, xxviii
- Wong, David, xxix
- Worden, Frederic G., 30
- Yamamura, Henry, xvii–xviii
- Yeast two-hybrid screening, 330, 372, 418
- Young, Anne B., xvii–xviii
- Zofran (ondansetron), xxxi